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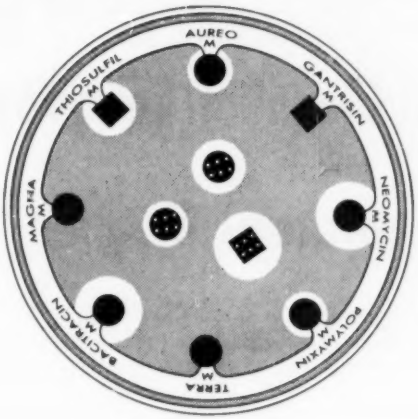

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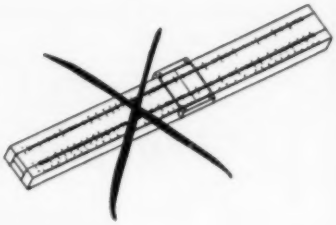

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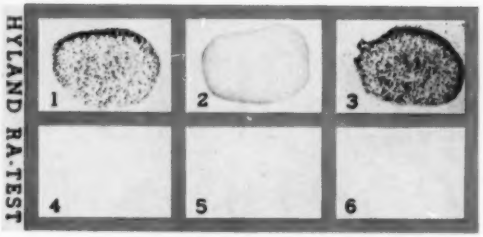

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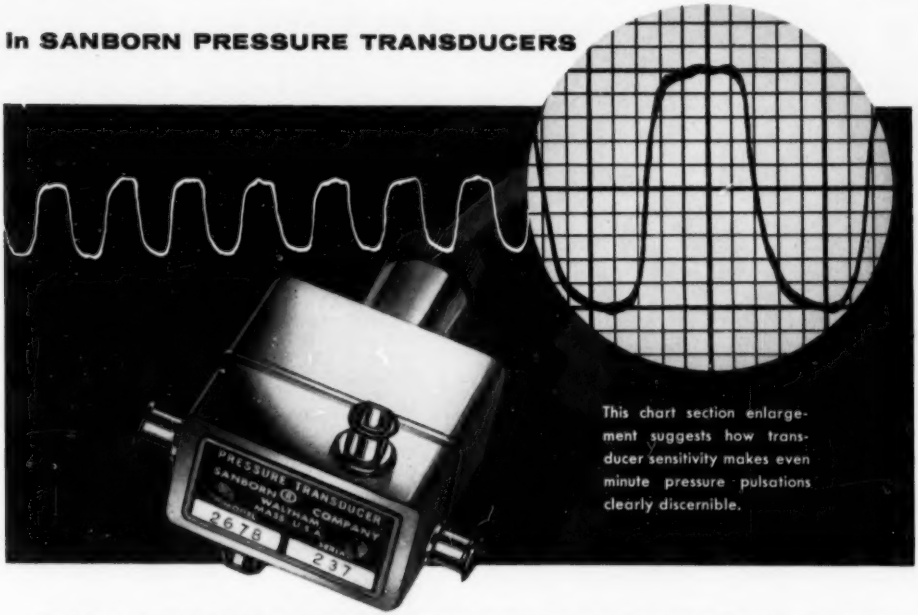

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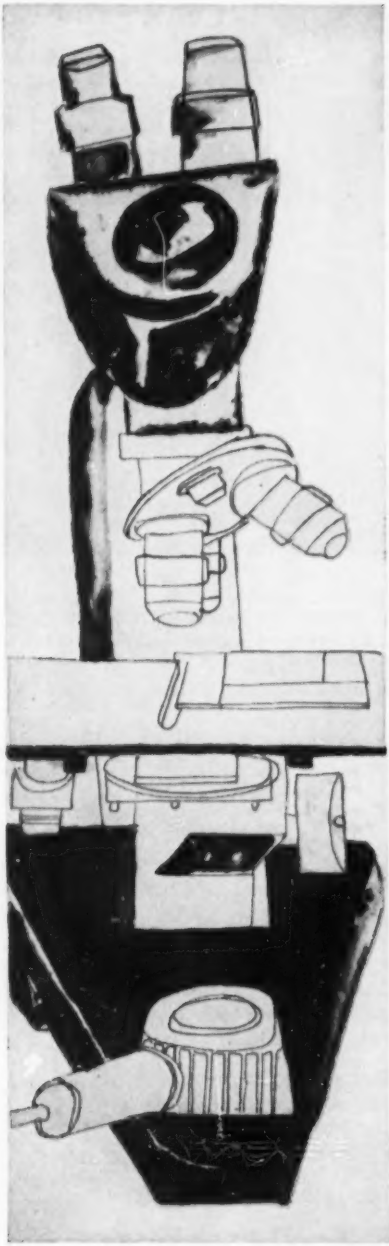

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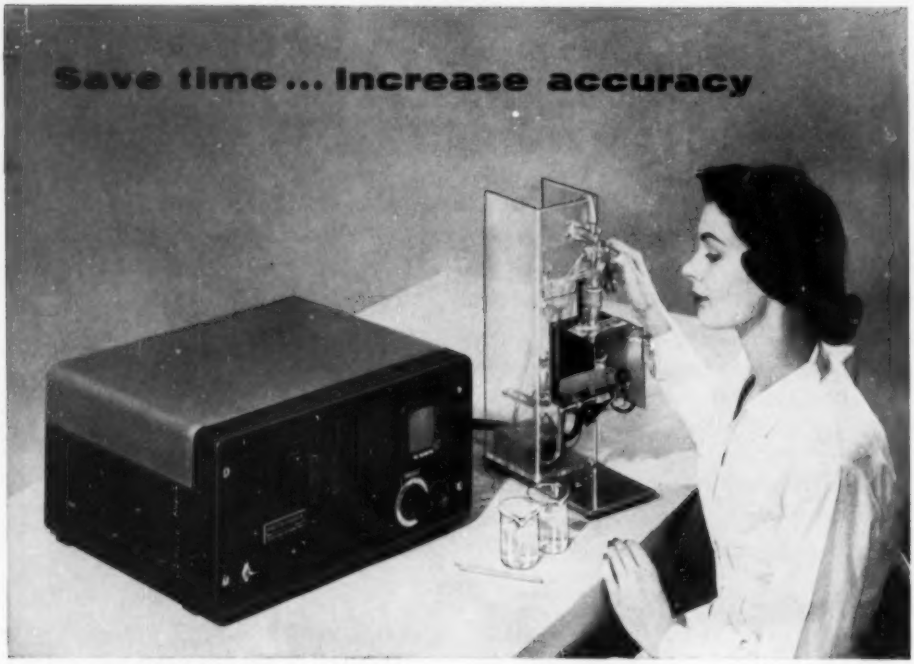

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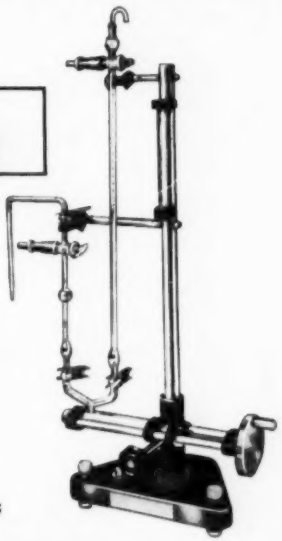

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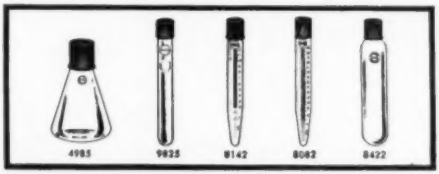

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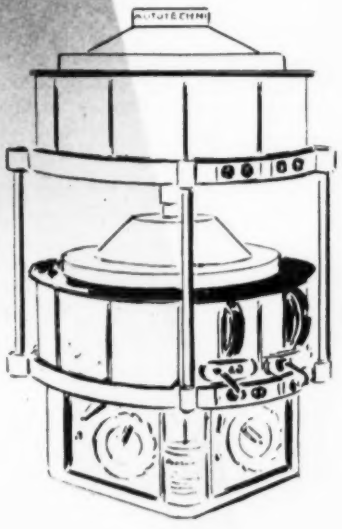

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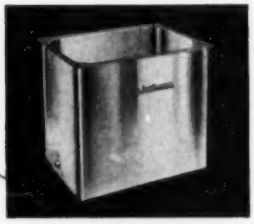

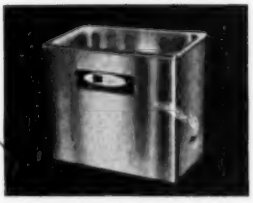

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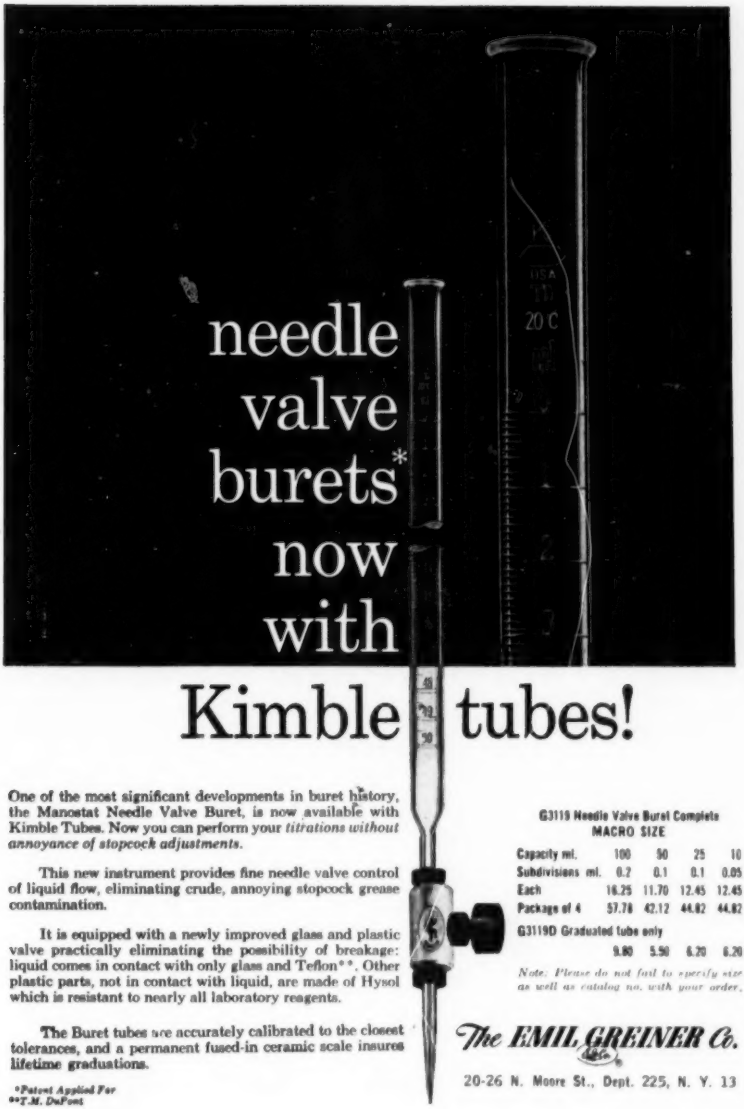

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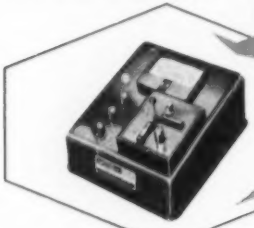
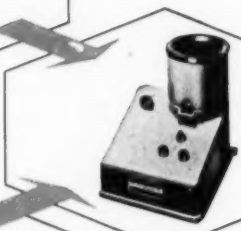
The Junior Spectrophotometer integrates with the Flame Photometer to provide complete spectrophotometry **plus** flame photometry.





The Universal Spectrophotometer integrates with the Photofluorometer to extend fluorimetry into the range of trace analysis.

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Each of these Coleman instruments is a respected and valued tool of the medical and clinical laboratory. Each performs its analytical function with precision and dispatch. Moreover, these ***Coleman instruments can be combined into analytical teams*** that vastly increase the scope and versatility of the analytical laboratory, without added cost and without interference with their basic uses.

New literature available:

1. The Coleman Universal Spectrophotometer . . . . Bulletin B-241
2. The Coleman Flame Photometer . . . . . Bulletin B-242
3. The Coleman Junior Spectrophotometer . . . . . Bulletin B-240A
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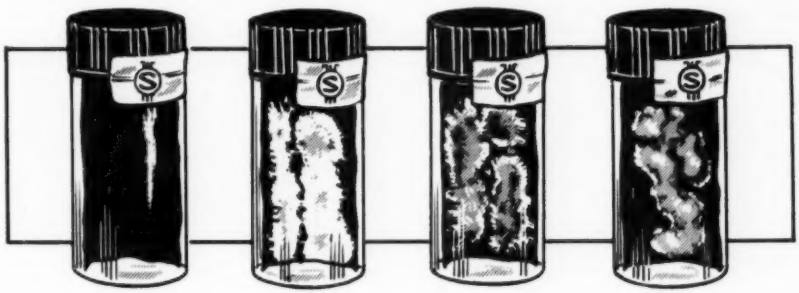

Uninoculated *T. gypseum* *T. purpureum* *C. albicans*

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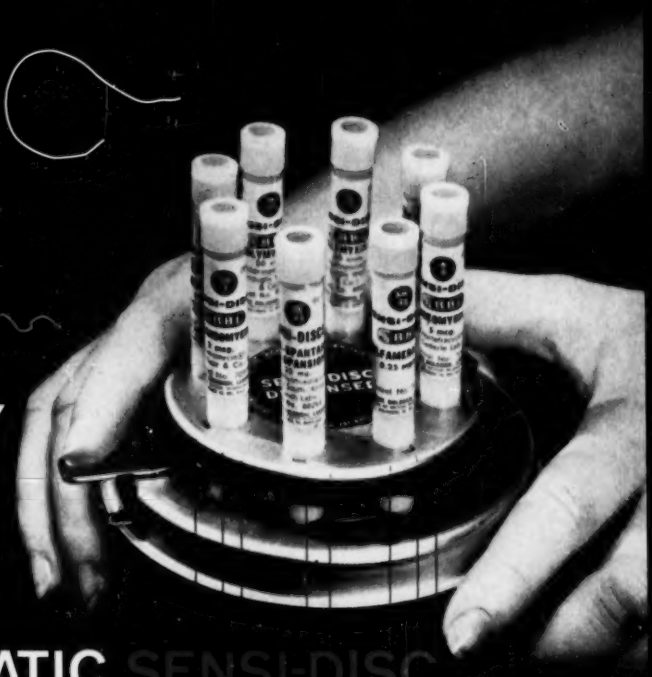

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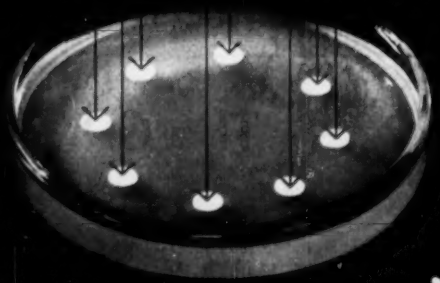

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At the crossroads

Our devotion to *Chemical Abstracts* nomenclature for our line of organic reagents is commendable, justifiable, and sometimes fatuously self-sacrificing. We are holier than most chemical houses. Why, we know some producers of dyestuffs very useful in the laboratory who *refuse* orders written in the systematic nomenclature, much less encourage them!

Between trademarks—which specify only commercial origin for merchandise otherwise named as to kind—and systematic but pedantic chemical names lies the convenient middle ground of trivial names (trivial: three roads, i.e., the fork where the people meet to chat, get it?). Some are trademarks abandoned by their owners, some are pronounceable combinations of letters from the systematic name, some are little amateur ads concocted by enthusiastic chemists who discovered the uses.

We keep a private book which lists thousands of different names for our 3700 odd compounds. It has suddenly struck us that a small fractional distillation of this book, comprising a few of the names neither cross-referenced in our catalog nor familiar to everybody gabbing at the crossroads, should be put in the hands of any person interested enough to ask. He gets it from Distillation Products Industries, Eastman Organic Chemicals Department, Rochester 3, N. Y. (Division of Eastman Kodak Company). To use it the way we want him to, he must also have a copy of Eastman Organic Chemicals, List No. 41, which gives prices.

A talisman from New Haven

We manufacture 16mm motion picture projectors and film. The projectors are sold by audio-visual dealers, to be found in many cities. The film is purchased by motion picture producers, who place suitable images on it, so that there shall be some further purpose served by running it through the projectors sold by the audio-visual dealers. Our intentions are direct and praiseworthy. We want large numbers of people to visit the audio-visual dealers, to discuss with them such matters as the advantage of a 25-watt amplifier over a 15-watt amplifier, and to conclude each such visit with a substantial order for each of the four current models of *Kodak Pageant Sound Projectors.*

Finding ourselves in mid-20th century, we have to proceed toward these simple objectives by sophisticated routes. We use more than mere words for persuasion. We employ graphic communication. From the Yale News Bureau, we acquire a photograph of their serene campus.

We assure the Bureau that no endorsement of us or our works is to be implied. The photograph serves only as a visual symbol of scientific objectivity.

Next we establish why we picture Yale instead of some other equally serene haven of objectivity. Yale was the site of two important conferences at which learned psychologists and successful communicators met and scrutinized the obstacles to wider and wiser use of films, slides, sound filmstrips, etc., in conveying information, skills, or attitudes from one group of heads to another. A second Yale angle is that two of its professors of psychology have prepared a scholarly evaluation of scientific principles for maximum learning from motion pictures and other audio-visual media. In the native patois of their discipline they say that movies are fine for teaching and suggest lines of research on how to improve them even further than they have already been improved by all the research they cite.

Their views and those of the conferees are given in a book entitled "Graphic Communication—and the Crisis in Education," obtainable from the Department of Audio-Visual Instruction of the National Education Association, 1201 16th Street, N.W., Washington 6, D.C. We suggest the hard-cover edition at $2. It makes just the right noise when slapped down at the opposite end of the table from any finance committee member who insists on something scientific that he can take home in his briefcase.

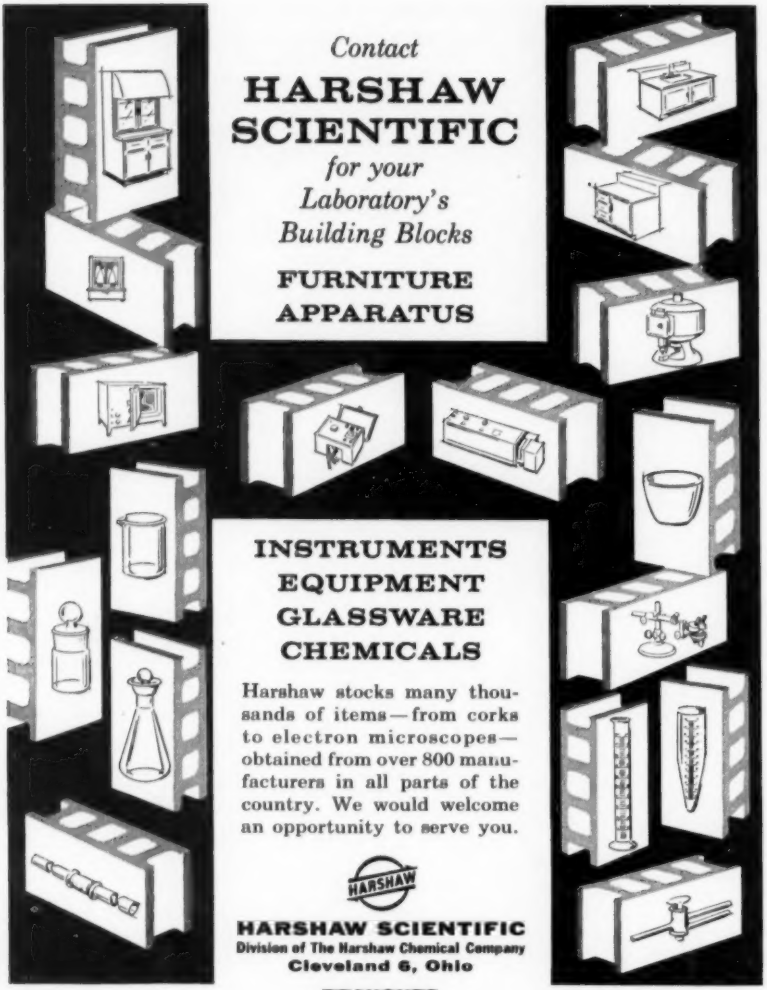
The wordy vitamin

In the three years 1955 through 1957, there have appeared to our knowledge 119 papers in the world's scientific literature on the occurrence and distribution of vitamin E, 60 on the determination of vitamin E, 148 on its chemistry, 362 on its relation to physiology and pathology, 85 on its pharmacology, 275 on its role in nutrition and metabolism, and 335 on its medical and therapeutic uses. There has been an international congress on vitamin E in Venice, and the vitamin has been definitely related to the hatchability of turkey eggs.

Because we like to think of ourselves as a potent force in the investigation and commercial production of vitamin E, one or the other of us has, with varying degrees of belief, read every one of these papers. We have subsequently prepared an annotated bibliography of them for distribution by the National Vitamin Foundation, 149 East 78th Street, New York 21, N. Y., the fourth such we have done. If you have use for a copy, you can state your case to the Foundation.

Now that it is printed, we have at least one regret. On page 56 we see the word "preventative." Rhetoricians deplore the word "preventative."

Kodak



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One Manufacturer's Opinion

A Welcome Borrowing

Observing the success of quality control in industry, professional groups are asking why quality control techniques should not be applied to other areas. Recent events point up demands by leading scientists for this application—especially to those areas dealing with the safeguarding of human life.

Aside from the particular product or service offered by a manufacturer, long-term success is, after all, dependent on the guarantee of reliability and quality offered the consumer. Knowing this, industry has developed rigid quality control over production. Extensive testing, taking weeks or months, is undertaken before release of a product run. This testing serves a dual purpose. First, efficiency in manufacturing is maintained and guarded; secondly, the product or service offered is kept at a standard to satisfy the discriminating public.

In the clinical laboratory, advances made in specific instrumentation, methods, technique, and diagnostic reagents have, until recent years, greatly exceeded the overall quality control practiced over biochemical determinations.

It is not surprising that modern advances and antiquated control are functioning side by side in laboratory testing. *Adequate control measures have not been available.*

Now, two major steps have been taken to fill this void. First, the application of statistical measures of precision and of accuracy, and the routine use of the specific deviation yardstick are becoming widespread *in revealing* laboratory efficiency problems. Secondly, the common usage of two important standards-in-serum for biochemical testing, Versatol (normal) and Versatol-A (abnormal), with known absolute values in the normal and abnormal range, meet the demands of advanced laboratory analyses.

Combined with technical skill, these safeguards over laboratory testing add welcome new chapters to accuracy in clinical chemistry.

Quality control in clinical chemistry featured at scientific meeting

Clinical Pathologists, Chemists Eye Application of Industrial Advances to Laboratory

New York, N. Y., Nov. 12, 1958. Interest in clinical laboratory quality control was heightened today when, the second time this month, this subject was covered at the Symposium on Laboratory Instrumentation at the Hotel New Yorker. Stephen J. Koziol of Warner-Chilcott, in a paper on "Recent Developments in Increasing the Efficiency of Clinical Analyses", stated the approach used by his firm:

"The third ... part deals with the objective standards and the objective controls which may be applied by the conscientious laboratory worker – to assure that neither human fatigue nor instrument fatigue are giving rise to clinically invalid results. Reference standards – closely duplicating both normal and pathological clinical chemistry specimens ... have been developed to aid quality control within the clinical laboratory itself."

Two reference standards, Versatol and Versatol-A, which closely duplicate both normal and pathological clinical chemistry specimens have been developed to aid quality control within the laboratory itself.

Earlier, in this same Symposium, pioneers in microchemistry recommended Versatol and Versatol-A for the *standardization* of the newest microchemical methods.

Quality Control in Clinical Chemistry was a featured presentation earlier this month at the American Association of Clinical Pathologists meeting in Chicago. The use of freeze-dried reagents *like* Versatol and Versatol-A was recommended for routine daily control.

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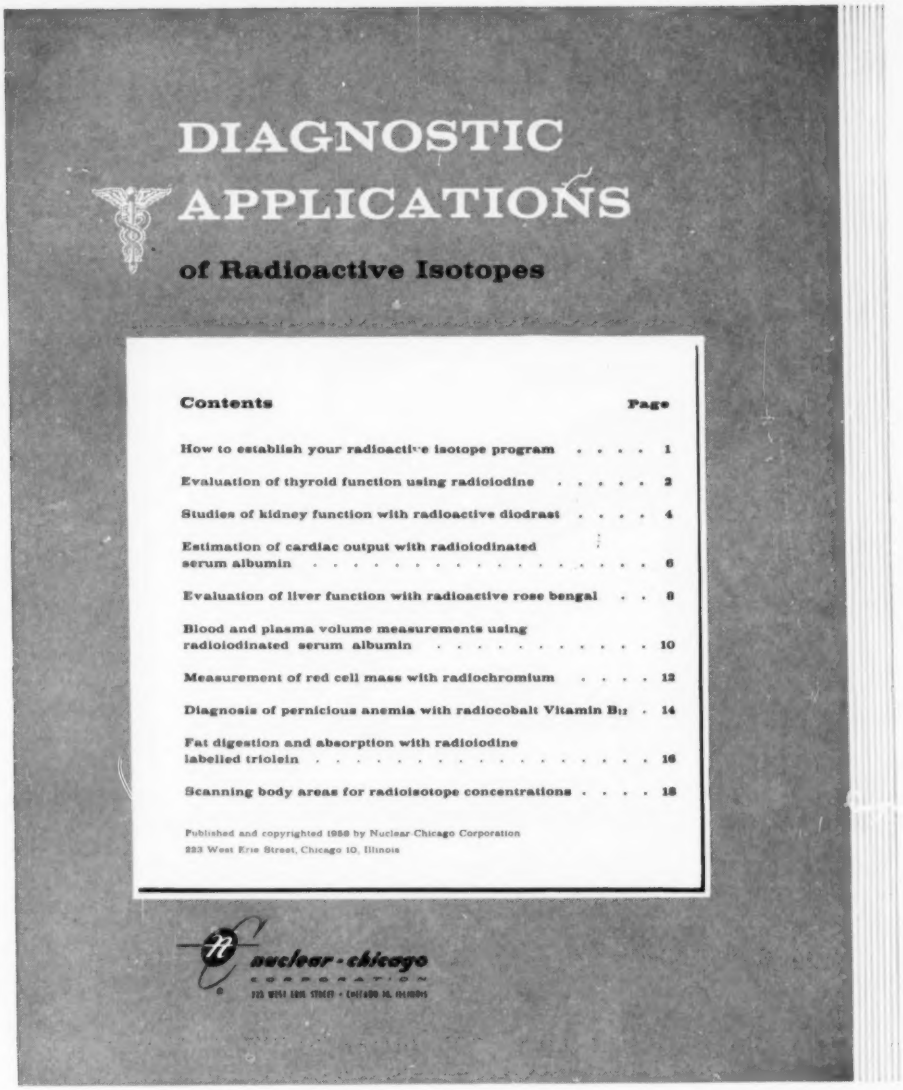
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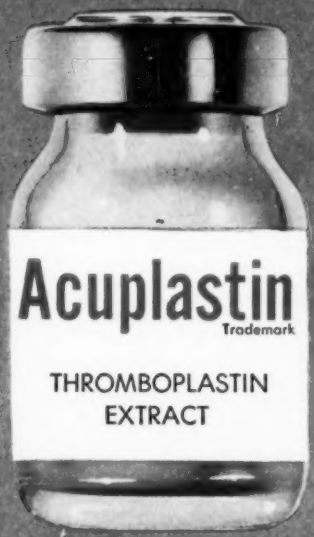

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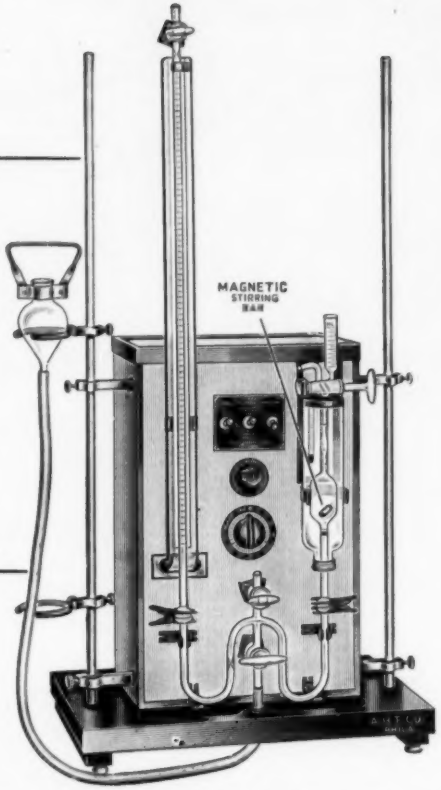

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The Journal of Laboratory and Clinical Medicine

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LETTER FROM THE EDITORS

Dr. Richard B. Capps, Dr. Wendell H. Hall, and Dr. Richard W. Vilter have completed their six-year terms on the Board of Editors. On behalf of the Central Society for Clinical Research and the Editorial Board, we wish to express our appreciation for their valuable services.

Dr. Robert H. Furman, Dr. Robert M. Kark, and Dr. Gordon Meiklejohn have accepted appointments and Dr. Oscar D. Ratnoff has accepted reappointment on the Board of Editors.

During the past year the Editors and Board members have called upon many of their associates and colleagues in other universities to aid them in the evaluation of manuscripts. Those who have participated in this capacity in the past year are:

Abell, Murray R.
Alving, Alf. S.
Baker, Burton L.
Barclay, William R.
Barlow, Charles
Bauer, Jere M.
Bernstein, Isadore A.
Berson, Soloman
Bishop, Ronald C.
Block, Walter D.
Bollett, Alfred J.
Braude, Abraham I.
Bricker, Neal S.
Brown, Gordon
Carr, Edward A., Jr.
Cartwright, G. E.
Chandler, Joseph P.
Christensen, Halvor N.
Christian, Charles
Christman, Adam A.
Conn, J. W.
Coon, Minor J.
Corcoran, A. C.
Darby, William
Davenport, Fred M.
Davey, Winthrop N.
Dennis, Edward
Dingle, John
Donaldson, Virginia
Duff, Ivan F.
Dunstan, Harriet
Eckstein, Henry C.
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Flink, Edmund B.
Fowler, Willis M.
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Freinkel, Norbert
French, A. James
French, Arthur B.
Furman, Robert
Glazer, Helen
Graham, Bruce
Gurney, Clifford W.
Hagen, Paul S.
Hanke, Martin
Haserick, John
Henley, Keith
Hennessy, Albert V.
Hilleman, Maurice R.
Hoffbauer, F. W.
Hoobler, Sibley W.
Huff, Rex L.
Isselbacker, K. J.
Jacobson, Leon O.
Johnston, Franklin D.
Kappas, Attallah
Kark, Robert M.
Kipnis, David
Kirsner, Joseph B.
Knauff, Raymond
Korst, Donald R.
Laszlo, Daniel
Lepper, Mark
Lewis, Jessica
Lewis, Lena
Louis, Laurence
Lowry, Oliver H.
Manger, William
Margolius, Alvin, Jr.
Mason, Merle
Meyers, Muriel C.
Miller, George
Moloney, William C.
Moore, Carl V.
Morgan, Herbert R.
Mosbach, E. H.
Mueller, John F.
Nungester, Walter J.

Olson, Robert E.
Owen, Charles A.
Pearson, William N.
Pigman, Ward
Pollard, H. Marvin
Price, J. M.
Ragan, Charles
Reiffel, Leonard
Remington, Richard D.
Riley, Gardner
Roseman, Saul
Ross, Joseph F.
Rundles, Ralph W.
Scheinberg, I. Herbert
Schilling, Robert F.
Schneider, Richard C.
Schoch, Henry K.
Schubert, Maxwell
Seegers, Walter H.
Seegmiller, J. Edwin
Shulman, Lawrence E.
Shulman, Sidney
Smith, Audrey
Solari, Arthur J.
Spencer, Herta
Sprague, Randall G.
Stollerman, Gene H.
Streeten, David H. P.
Tourtellotte, Wallace W.
Vaughn, John H.
Vial, Burgess
Ward, Will W.
Watson, Cecil J.
Watson, Ernest H.
Weller, John M.
Wiener, Alexander S.
Wilde, Walter S.
Wilkinson, Charles F.
Willis, Park W., III
Wilson, James L.
Wintrobe, Max
Witebsky, Ernest
Woods, Lauren
Wu, Chung
Zieve, Leslie
Zilversmit, D. B.
Zinneman, Horace
Zuelzer, Wolf W.

We wish to take this opportunity to thank the past and present members of the Editorial Board for their excellent services to the JOURNAL and to the Central Society for Clinical Research. We would also like to express our appreciation for the cooperation received from The C. V. Mosby Company.

WILLIAM D. ROBINSON, M.D., EDITOR
WILLIAM H. BEIRWALTES, M.D., ASSOCIATE EDITOR

THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

An Examination of Title

Thornton Scott, M.D.
Lexington, Ky.

FELLOW members and most welcome guests, the honor of addressing you is the highest of my professional life. At the moment of my nomination and election as Vice-President (of this Society), my initial reaction was that of anesthetized astonishment. When the anesthesia began to wear off, I began, as one might, to wonder why and to rationalize and, perhaps erroneously, to imagine that there might be, after all, some reason for selecting me, an impure scientist at best. This led me to reflections on impurity in general. Is it a curse or a blessing? Perhaps it is a bit of each. By definition it must be a mixture of the two, for anything totally impure must be pure, as is that which it totally pure. The worthiness of purity, then, must be judged in its context. Impurities are most disturbing in the analytic laboratory or in the turbines of a battleship, although highly desirable in the world of gastronomy and vitamins. Impurities in the atmosphere make a glorious sunset. Impurities distinguish the finest vintage wine from the alcohol of commerce, and fine old Bourbon from vodka.

Last year, I promised that the presidential address would be read by title only. This year I have immodestly reneged to the extent that it shall concern itself with an examination of title. The title given this Society by its founders, Central Society for Clinical Research, implies impurity. The founders did not call it the Central Society for Pure Research. They injected the word "clinical." Some of us have felt that the Society, in recent years, may have lost some of its pristine impurity. As a consequence of certain trends in American medicine which have developed during my medical lifetime, an unfortunate cleavage has arisen between the pure clinician and the pure investigator, between the practicing physician and the academician. Similar cleavages have arisen between the artist, the intellectual, and the man of affairs. Perhaps these result from lack of a preliminary basis of broad cultural education and an excess of technology. In this regard, our colleagues in England and on the Continent are not so sorely afflicted. I should like to believe that those responsible for my addressing you were cognizant of my interest in research and the investigative method, while at the same time actively engaged in clinical practice. It is, therefore, my hope that this address may help in some way to heal this schism, which should never have existed, between pure science and clinical medicine. Identical principles must govern any proper study. Desire for truth and the

Presidential Address, Thirty-first Annual Meeting of the Central Society for Clinical Research, Chicago, Illinois, Oct. 31 and Nov. 1, 1958.

ability to recognize it; avoidance of self-deception or distortion of results; restraint of enthusiasm; and the basic principles of logic must underlie all of our intellectual efforts. Imagination and an alert, honest, and inquiring mind are as essential at the bedside as in the laboratory. Neglect of these principles can lead to the worst in armchair medicine as well as to the worst in potboiling, grant-consuming publications promulgated by "measuring worms" and what one might call "metrognomes."

I should like to cite briefly an example of what I consider fundamental and good clinical investigation, the work of a group at the United States Public Health Service Hospital in Lexington, Isbell, Fraser, and Wikler. In an elaborately controlled way, in two carefully planned studies, they addicted hitherto normal subjects to barbiturates and to alcohol. They plotted the course of addiction, its effect on cerebral physiology and psyche, and then observed the effects of abrupt withdrawal. Detailed observations of these effects can show us exciting vistas in the realms of psychiatry, pharmacology, and neurophysiology. By using organic agents and withdrawing them, they have produced grand mal, psychomotor and petit mal seizures, psychoses, neuroses, hallucinations, and hysterical reactions. Many of these manifestations had been hitherto considered purely functional; yet, they were produced not by the exhibition of an organic agent but by its withdrawal. Such studies as these could well give pause to the supernaturalists and intrauterine behaviorists, and give support to physiologic "unitarians." With regard to alcoholism they have shown that delirium tremens and other psychoses can be caused by the abrupt withdrawal of alcohol—a belief firmly held in previous generations, but forgotten in the wave of enthusiasm for vitamin deficiencies.

After this *divertimento,* we now come to the development section of our theme. After much discussion with members more widely experienced than I, it became evident that many consider that this Society has attained sufficient dignity and seniority to strike out in a new direction and develop a character more nearly its own. Many believe it is no longer necessary or desirable to compete with the various Federations for papers which are, in the main, methodological. Some believe we should combine some of the better aspects of the Association of American Physicians with the better characteristics of our own Society as it now stands. We could benefit from more active participation in the affairs of the Society by older and more experienced members, many of whom are on the emeritus list. Some of them were in at the birth and played an important part in its development. We should hold their interest and utilize their talents. Indeed, it would be well for the Society to elect one emeritus member and one active member to the council each year. Better balance could also be achieved by devoting one day to outstanding papers of a nonclinical nature, allowing sufficient time for clarification and development of ideas and fuller explanation for the benefit of those outside their narrow field of interest. The other session could be devoted to papers of a clinical nature. This might make it desirable to utilize Saturday afternoons to allow adequate discussion. In this

way, we could offer varied programs in a more leisurely fashion. These thoughts are offered for your consideration during the coming year. You are urged to discuss their merits and demerits.

In summary, we should examine the title of this Society and be reminded that it was founded by men who were primarily interested in medicine, both clinical and investigative. Our programs should maintain a more equable balance in this respect. We should use the talents of our more experienced members on the council and on the programs, to the end that we may enjoy what many of us have experienced at Atlantic City on many a May Monday, a hectic day of frantic but stimulating bewilderment, followed by a calm Tuesday of clinical and cultural edification.

CLINICAL AND EXPERIMENTAL

RENAL HEMODYNAMICS IN PATIENTS WITH COARCTATION OF THE AORTA

WALTER M. KIRKENDALL, M.D., JAMES W. CULBERTSON, M.D.,* AND JOHN W. ECKSTEIN, M.D.,**
IOWA CITY, IOWA

THE renal circulation has been studied frequently in patients with coarctation of the aorta to support or refute the hypothesis that the systemic arterial hypertension seen above the aortic constriction in such patients results from renal ischemia. There are conflicting reports of (a) significantly decreased renal blood flow and near-normal glomerular filtration rate,[2, 3] (b) slightly decreased renal blood flow and glomerular filtration rate,[4] and (c) normal values for both functions.[4-6] Only Friedman and associates[2] have found a marked decrease in renal blood flow to be a consistent finding in all of their patients (6) with coarctation. The variable results have been interpreted by different investigators to substantiate one or the other point of view concerning the possible presence and significance of renal ischemia in this condition. Although it has been pointed out by Werko and associates[7] that the demonstration of normal renal hemodynamics by current methods cannot exclude possible localized renal ischemia as a cause of the hypertension of coaretation patients, demonstration of normal kidney blood flow rates and normal arteriolar resistance in the renal circuit would serve to make generalized arteriolar constriction and, hence, significant sustained renal ischemia unlikely.

The postulate that there is generalized systemic arteriolar constriction in patients with coarctation of the aorta was proposed first by Steele[8] in 1941. He suggested that the observed hypertension was not caused entirely by mechanical obstruction to blood flow from the proximal aorta but resulted partially from a humoral vasoconstrictor, which probably was produced by renal ischemia. We have discussed this proposal in a previous report on the general hemodynamics and visceral circulation in 10 patients with coaretation of the aorta.[9]

From the Renal Laboratory, Cardiovascular Research Laboratories, and the Department of Internal Medicine, College of Medicine, State University of Iowa, and from the Medical Service, Veterans Administration Hospital, Iowa City, Iowa.

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Presented in part at the Twenty-Seventh Annual Meeting of the Central Society for Clinical Research, Chicago, Oct. 29 and 30, 1954.[1]

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*Present address: Department of Internal Medicine, College of Medicine, The University of Tennessee, Memphis, Tenn.

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The purpose of this paper is to report our renal physiologic findings in 21 patients (14 men and 7 women) with coarctation of the aorta and to examine these data for evidence favoring or opposing the hypothesis that generalized arteriolar constriction frequently causes the upper compartment hypertension seen in patients with this congenital vascular anomaly. Since the hemodynamic pattern may not be the same in all patients, and since conflicting reports on renal hemodynamics are more conspicuous than on other features of the disorder, we extended our own series of renal observations to 21 patients in the hope of encompassing the entire range of hemodynamic patterns for analysis in a single experimental series.

SUBJECTS AND METHODS

All 21 patients reported in this investigation had been admitted to the State University of Iowa General Hospital. Each had unequivocal clinical and laboratory signs of coarctation of the aorta, and 19 of them underwent corrective surgery after completion of research studies. Two patients, WOL and CLY, were unwilling to have the operation, although both were considered medically suitable despite the fact that WOL was relatively old (44 years).

Renal circulation was studied by means of standard plasma clearance techniques,[10] with the use of sodium para-aminohippurate (PAH) to measure effective renal plasma flow (C_{pah}). Glomerular filtration rate was measured by inulin clearance (C_{in}) in 19 patients and by sodium thiosulfate clearance[11] in 2 (GRO and CUP). Inulin determination in one patient (RYA) was made by Schreiner's modification of the Roe resorcinol method[12] and in the other 18 by a diphenylamine technique.[10] Satisfactory diuresis was promoted by oral administration of water to all patients. After appropriate priming, an equilibration infusion period of 35 to 45 minutes preceded each study. Venous blood was collected from the arm through an indwelling Unger needle 2 minutes before the mid-point of each urine collection period of approximately 14 minutes. Relatively constant plasma levels of the test drugs were maintained. All observations were made with plasma inulin in the range of 20 to 40 mg., sodium thiosulfate 20 to 40 mg., and PAH 1 to 3 mg. per 100 ml. Tubular excretory mass (Tm_{pah}) was measured in 3 patients with plasma PAH of 60 to 80 mg. per 100 ml. Each reported plasma clearance value is the average of at least 3 determinations in each patient and has been corrected to a standard surface area of 1.73 square meters. Effective renal blood flow was calculated from the effective renal plasma flow by the multiplication factor: $\frac{1}{1 - \text{hematocrit}}$.

Phasic and electrically integrated mean pressure levels were measured with capacitance manometers through indwelling needles in the brachial and femoral arteries and were registered by a direct-writing oscillograph,* unless otherwise indicated. Renal resistance values were calculated by the method of Gomez[13] from electrically integrated mean femoral arterial pressure in 17 patients. In patient FOR, a sphygmomanometric femoral arterial pressure was used for the calculation of renal resistance, the mean pressure being taken to be the diastolic pressure plus 43 per cent of the pulse pressure.[14] In 3 patients neither auscultatory nor direct femoral arterial pressure measurements were obtained; therefore, renal resistance values are not estimated for them.

Gomez's formulation for the estimation of segmental renal vascular resistance was used to demonstrate hemodynamic differences which might not be apparent otherwise. Since no characteristic or regular anatomic changes are known to occur in the kidneys of patients with coarctation, we believe that the use of this method of calculating renal resistance is as valid in these patients as it is in those with essential hypertension.[13] We recognize the assumptions required for application of these formulas to kidney function even in the absence

*Sanborn Company, Waltham, Mass.

TABLE I. HEMODYNAMIC DATA FROM PATIENTS WITH COARCTATION OF THE AORTA

PATIENT	AGE	SURFACE AREA (M.²)	HEMATOCRIT (PER CENT)	BRACHIAL ARTERIAL PRESSURE		FEMORAL ARTERIAL PRESSURE		AVERAGE GLOMERULAR FILTRATION RATE (ML./MIN./1.73 M.²)	AVERAGE RENAL PLASMA FLOW RATE (ML./MIN./1.73 M.²)	AVERAGE RENAL BLOOD FLOW RATE (ML./MIN./1.73 M.²)	AVERAGE FILTRATION FRACTION (DECIMAL)
				PHASIC (mm. Hg)	MEAN* (mm. Hg)	PHASIC (mm. Hg)	MEAN* (mm. Hg)				
A. *Men*											
GRO	18	1.88	44.0	165/90	115	120/75	85	226	971	1,734	0.233
SCH	20	1.83	45.0	175/125	147	105/90	97	149	698	1,269	0.213
REI	15	1.61	40.0	125/70	88	90/70	80	147	758	1,263	0.194
WOL	44	1.96	44.1	205/100	135	115/80	95	142	624	1,142	0.228
RYA	24	1.95	44.5	155/90	105			128	631	1,127	0.203
WEI	27	1.72	44.0	250/100	150	125/95	105	116	586	1,064	0.198
DOW	32	1.81	47.0	200/115	150	125/95	110	120	551	1,040	0.218
CUP	44	1.93	46.0	165/90	120	105/80	85	121	553	1,005	0.219
HEI	19	1.69	36.6	160/95	120	105/75	85	116	626	986	0.185
TIG	18	2.01	43.8	180/80	110	110/75	90	125	542	970	0.231
HEN	12	1.35	36.0	180/105	145			138	565	885	0.244
ROB	20	1.92	45.0	157/105		110/90	100	141	466	847	0.303
MAT	15	1.85	43.0	180/95	125	120/90	100	107	422	740	0.255
Average	24	1.81	43.0	177/97	126	112/83	94	137	615	1,082	0.225
Standard deviation	10	0.18	3.4	30/13	20	11/9	10	30	139	108	0.031
B. *Women*											
FOR	15	1.38	39.0	184/120†	148†	102/84†	99†	120	627	1,028	0.191
FAH	18	1.62	39.0	150/80	105			115	549	900	0.209
BOL	19	1.84	40.0	200/110	140	125/100	105	119	530	884	0.225
CLY	30	1.58	37.0	175/100	135	130/90	110	117	543	862	0.215
Average	21	1.61	38.8	177/103	132	119/91	105	118	562	919	0.210
Standard deviation	7	0.19	1.3	21/17	19	15/8	6	2	44	75	0.014

*Electrically integrated.

†Sphygmomanometric pressure and calculated mean pressure. At operation intraaortic pressure above the constriction was 170/100 mm. Hg, with a mean of 120; while below the constriction the pressure was 90/75, with a mean of 85.

of demonstrable organic vascular disease. The problem of validity is much greater when the Gomez formulas are used to calculate segmental vascular resistance values in the presence of congestive heart failure.*

To provide information for comparison with our observations in human aortic coarctation, we averaged similar renal hemodynamic data obtained in our laboratory from 25 normotensive men without evidence of cardiovascular or renal disease and from 29 male patients with mild or moderate essential hypertension. Inulin clearance was used to measure glomerular filtration rate in each instance. Patients were considered to be hypertensive if the average of 4 systemic arterial diastolic pressure readings on each of 3 successive days just prior to the renal clearance study was 90 mm. Hg or more. All blood pressure records were made with the subjects resting in a recumbent position. None of the hypertensive control patients had evidence of congestive heart failure, myocardial or cerebral infarction, azotemia, or primary renal disease. Twenty-one of the normotensive control men without cardiovascular or renal disease were drawn from the hospital and outpatient clinical population, and careful examination revealed no significant disease in any of them; the remaining 4 were healthy medical students. The men in both control groups were below the age of 50, and all were studied during the same total elapsed time period during which the coarctation data were being accumulated. Calculation of renal vascular resistance in these normal subjects and hypertensive patients was based on mean† systemic arterial pressure calculated from brachial cuff measurements and on an assumed renal venous pressure of 10 mm. Hg, since none of these individuals was in heart failure. The same renal venous pressure was used for patients in whom distal occluded pulmonary artery (''capillary'') pressure was normal and in those compensated patients in whom cardiac catheterization was not done. Whenever cardiac catheterization was omitted, great care was taken to make certain that the patient did not have clinical signs of congestive heart failure. Specifically, in these patients there was no evidence of increased systemic venous pressure, and no hepatojugular reflux could be demonstrated; nor was there any evidence of pulmonary congestion, clinically or radiographically. In the 4 patients with congestive heart failure the renal interstitial pressure and the renal venous pressure were assumed to be the same. A standard value of 7 Gm. per 100 ml. was used for total plasma protein in all calculations except those involving patients with congestive heart failure, where observed plasma protein values were used. The average normal finding in our clinical biochemistry laboratory is 7 Gm., and we verified this in a majority of the patients with coarctation. Statistical analyses of the various functions were made by standard methods.[15]

Measurements of minute volume cardiac output and total oxygen consumption, hepatic blood flow and splanchnic oxygen consumption, and arterial pressure levels in the upper and lower systemic compartments were made within 24 to 48 hours of the renal studies in 10 patients. This information, with calculation of total systemic (i.e., upper compartment) arterial and splanchnic arterial resistance values, has been reported.[9]

RESULTS

I. *Thirteen Men With Coarctation of the Aorta Not in Congestive Heart Failure (Table 1).*—

A. *Comparison with normotensive men:* Patients with coarctation of the aorta were 7 years younger on the average and had significantly higher pressure in the upper systemic arterial compartment. In the lower compartment the measurements reflect the characteristic damping of the pressure pulse wave in

*Not only is renal interstitial pressure elevated to an unknown degree in heart failure patients, but obvious organic changes occur in chronic congestive failure which render the interpretation of the data ever more difficult. Although we present the calculated resistance data for the 4 coarctation patients in congestive heart failure, we do so for purposes of comparison only and make no claim for validity.

†Diastolic pressure plus 43 per cent of pulse pressure.

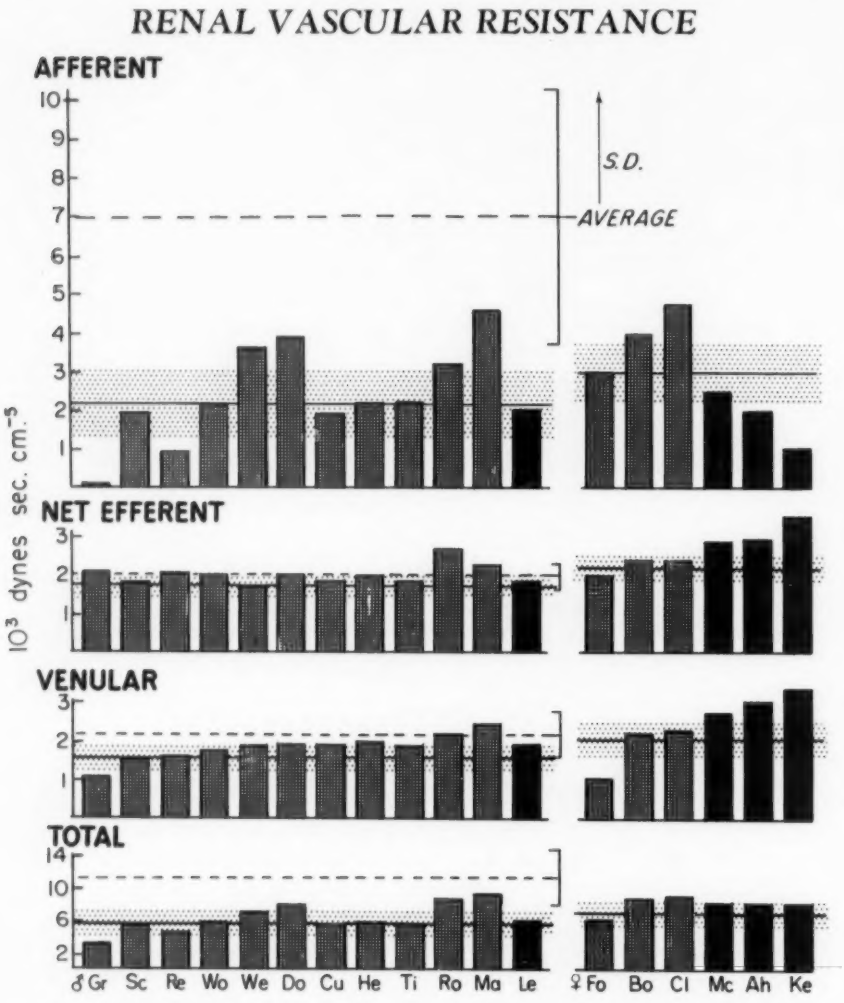

Fig. 1.—Segmental and total renal vascular resistance in 18 patients with coarctation of aorta. Twelve men are grouped on the left and 6 women on the right. In each group the patients are arranged from left to right in order of decreasing renal blood flow. The patients in congestive heart failure (solid black vertical bars) are placed to the right of their respective uncomplicated groups. In each frame the solid horizontal line represents the mean value and the width of the horizontal stippled area equals two standard deviations for comparable groups of normotensive men and women. For comparison with the men having coarctation of the aorta, corresponding data from 29 men with essential arterial hypertension are depicted by a broken line to indicate the average. One deviation on either side of the average is shown by the bracket. We lacked adequate data for making a similar comparison with women having essential hypertension.

systolic and diastolic phases for the coarctation patients, but their mean pressure is almost identical with that of the control group. There was a statistically significant increase in the filtration fraction in the coarctation group, as well as a similar increase in the net efferent renal vascular resistance (Table II and Fig. 1). In other functions, measured or calculated, there was no significant difference between the two groups.

One of the factors responsible for the increase in average filtration fraction and average net efferent arteriolar resistance was the relatively and absolutely high glomerular filtration rate of GRO (Table I). It is possible that this value is falsely high, since sodium thiosulfate was used for the determination. In our hands the thiosulfate:inulin ratio has varied from 0.9 to 1.2, and there are reports of even higher values.[16] When tests of renal function were repeated 6 months after surgical correction of the coarctation in this patient, the glomerular filtration rate (measured with inulin) had fallen from 226 to 164 ml. per minute, while the renal plasma flow had risen from 971 to 1,133 ml. per minute.

Tm_{pah} was measured in 2 of these coarctation patients and was found to be 92 mg. per minute for WOL, with $C_{pah}:Tm_{pah}$ and $C_{in}:Tm_{pah}$ ratios of 6.8 and 1.5, respectively; and 78 mg. per minute for RYA, with ratios of 8.1 and 1.6, respectively. These values are normal in our laboratory and in published reports.[17]

B. *Comparison with male hypertensive patients:* The coarctation patients were younger by 16 years on the average and had significantly lower average hematocrit than did the hypertensive patients (Table II). They had significantly lower brachial arterial diastolic pressure but comparable systolic and mean pressure levels. Femoral arterial pressure in all phases, however, was very significantly lower in the coarctation group. Glomerular filtration rate and renal plasma flow were significantly higher in the coarctation group, but renal blood flow was not increased proportionately (reflecting the influence of the higher hematocrit of the hypertensive group). The highly significant difference in total renal vascular resistance between the two groups (Table II and Fig. 1) is caused by the excessively high afferent arteriolar resistance in the essential hypertensives. The renal vascular resistance pattern in the coarctation patients, by sharp contrast, is remarkably similar to that of the normotensive men. Equally striking is the fact that the normotensive men and hypertensive men showed highly significant differences between these two control groups in all of the hemodynamic functions tested except glomerular filtration rate (Table II).

II. *Women With Coarctation of the Aorta, Not in Congestive Heart Failure (Table I).*—The renal function measurements in these 4 patients with coarctation of the aorta have been compared with the 17 normal women reported by Goldring and associates[18] and by Smith and associates.[19] The mean age of our women with coarctation was lower than that of this control group (21 versus 33 years); but the average surface area was comparable (1.61 versus

TABLE II. COMPARISON OF PHYSICAL CHARACTERISTICS, ARTERIAL BLOOD PRESSURE, AND RENAL HYPERTENSION AND

FUNCTION*	UNIT	COARCTATION PATIENTS		NORMOTENSIVE SUBJECTS	
		MEAN	S. D.	MEAN†	S. D.†
Number		13		25	
Age	Years	24	10	31	8
Surface area	$M.^2$	1.81	0.18	1.84	0.21
Hematocrit	Decimal	0.43	0.03	0.45	0.10
Arterial pressure					
Brachial systolic	mm. Hg	177	30	121	9
Brachial diastolic	mm. Hg	97	13	76	8
Brachial mean	mm. Hg	126‡	20	95	8
Femoral systolic	mm. Hg	112§	11	(121)	(9)
Femoral diastolic	mm. Hg	83§	9	(76)	(8)
Femoral mean	mm. Hg	94§	10	(95)	(8)
GFR	ml./min./1.73 $M.^2$	137	30	127	16
RPF	ml./min./1.73 $M.^2$	615	139	674	41
RBF	ml./min./1.73 $M.^2$	1,082	108	1,226	250
FF	Decimal	0.225	0.031	0.192	0.031
RA	10^3 dynes sec. $cm.^{-5}$	2.461§	1.311	2.231	0.869
R'E	10^3 dynes sec. $cm.^{-5}$	2.091§	0.273	1.736	0.299
RV	10^3 dynes sec. $cm.^{-5}$	1.888§	0.406	1.686	0.313
R	10^3 dynes sec. $cm.^{-5}$	6.440§	1.763	5.657	1.242

*Key to abbreviations: GFR = average glomerular filtration rate; RPF = average effective renal plasma flow; RBF = average effective renal blood flow; FF = average filtration fraction; RA = renal afferent arteriolar resistance; R'E = renal net efferent resistance; RV = renal venular resistance; R = total renal vascular resistance.

1.63 $M.^2$), as was the mean hematocrit (38.8 versus 39.5 per cent). Mean glomerular filtration rate was almost identical (118 versus 117 ml. per minute) in the 2 groups. The estimated renal plasma flow, 562 (PAH) versus 594 (Diodrast) ml. per minute, and the renal blood flow, 919 versus 982 ml. per minute, were higher in the normal control group; nevertheless, the average values for the coarctation women were well within the normal range (594 ± 102 and 982 ± 184). The filtration fraction was slightly higher in the coarctation group (0.21 versus 0.20).

In the calculation of renal resistance from the normal blood flow figures reported by Goldring[18] and Smith[19] and their associates each woman was assumed to have had a plasma protein level of 7 Gm. per 100 ml., a renal venous pressure of 10 mm. Hg, and a mean brachial arterial pressure of 95 mm. Hg. The last figure was used because it was the approximate value quoted by Master and associates[20] for women of this average age in the general population. Fig. 1 and Table IV show the renal vascular resistance values of the two groups.

III. *Patients With Coarctation of the Aorta and Congestive Heart Failure (Table III).*—Three of the 4 patients in this group were women. All of them had mild degrees of clinical myocardial decompensation, although KEN had ankle edema and a distal occluded pulmonary artery ("capillary") pressure of 28 mm. Hg when the renal studies were made. AHL had a pulmonary "capillary" pressure of 26 mm. Hg, an arteriovenous oxygen difference of 9.0 ml. per 100 ml. and a cardiac index of 2.7 liters per minute. MeC's systemic venous pressure measured at the right antecubital area was 205 mm. H_2O. She had, also, cardiomegaly and mild orthopnea. LED, the only man in this group,

HEMODYNAMIC DATA IN MALE PATIENTS WITH COARCTATION OF THE AORTA AND ESSENTIAL ARTERIAL IN NORMAL MEN

HYPERTENSIVE PATIENTS		COARCTATION PATIENTS VERSUS NORMOTENSIVE SUBJECTS		COARCTATION PATIENTS VERSUS HYPERTENSIVE PATIENTS		HYPERTENSIVE PATIENTS VERSUS NORMOTENSIVE SUBJECTS	
MEAN†	S. D.†	t	p	t	p	t	p
29							
40	7	2.282	< 0.05	5.816	< 0.001	4.342	< 0.001
1.93	0.18	0.429	< 0.7	1.967	< 0.1	2.037	< 0.05
0.46	0.03	0.690	< 0.5	2.727	< 0.01	0.526	< 0.7
168	28	8.593	< 0.001	0.918	< 0.4	7.920	< 0.001
110	14	3.214	< 0.01	2.346	< 0.05	10.753	< 0.001
135	20	6.547	< 0.001	1.229	< 0.3	9.229	< 0.001
(168)	(28)	(2.499)	(< 0.02)	(6.306)	(< 0.001)		
(110)	(14)	(2.263)	(< 0.05)	(5.815)	(< 0.001)		
(135)	(20)	(0.312)	(< 0.8)	(4.752)	(< 0.001)		
118	23	1.300	< 0.3	2.188	< 0.05	1.618	< 0.2
515	125	1.912	< 0.1	2.263	< 0.05	6.250	< 0.001
952	251	1.926	< 0.1	1.753	< 0.1	3.946	< 0.001
0.233	0.032	3.007	< 0.01	0.749	< 0.5	5.125	< 0.001
7.043	3.241	0.604	< 0.6	4.440	< 0.001	7.097	< 0.001
2.099	0.352	3.278	< 0.01	0.071	> 0.9	3.989	< 0.001
2.209	0.575	1.581	< 0.2	1.662	< 0.2	3.992	< 0.001
11.350	3.870	1.441	< 0.2	3.963	< 0.001	6.943	< 0.001

†A tabular record of the original data from which these means and standard deviations were derived has been deposited as Document No. 5689 with the ADI Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington 25, D. C. A copy may be secured by citing the document number and by remitting $1.25 for photoprints or for 35 mm. microfilm. Advance payment is required. Make checks or money orders payable to: Chief, Photoduplication Service, Library of Congress.

‡Number = 12.

§Number = 11.

The figures in parentheses = brachial arterial blood pressure used for hypertensive patients and normal men.

had only a slightly elevated distal occluded pulmonary artery mean pressure (13 mm. Hg), but this arteriovenous oxygen difference was increased to 5.2 ml. per 100 ml., and his cardiac index was only 2.24 liters per minute. Because of these findings, we considered this patient to have incipient congestive failure although his renal function findings did not deviate far from average normal values (Tables III and IV). He represents an early transitional stage from the compensated to the moderately severe congestive failure group.

All of these heart failure patients had decreased renal plasma flow, normal glomerular filtration rate, and high filtration fraction—as well as increased postglomerular (net efferent and venular) renal vascular resistance—when compared with normal subjects. One should note that the mean femoral arterial blood pressure was low in this group (Tables I and III). Tm_{pah} in KEN was reduced to 45 mg. per minute; $C_{pah}:Tm_{pah}$ was 8.4, and $C_{in}:Tm_{pah}$ was 2.9. The latter ratio is high, indicating that the glomerular filtration rate was maintained better than was the ability to excrete sodium para-aminohippurate and showing that adequate glomerular function was preserved while tubular function was impaired. These hemodynamic characteristics are similar to those of patients in mild congestive heart failure from other causes, although glomerular filtration rate in this group was maintained unusually well.

DISCUSSION

Only the compensated male coarctation group was used for comparison with normal subjects and hypertensive patients for statistical tests because of the

TABLE III. HEMODYNAMIC DATA FROM PATIENTS WITH COARCTATION OF THE AORTA AND CONGESTIVE HEART FAILURE

PATIENT	AGE	SURFACE AREA (M.²)	HEMATOCRIT (PER CENT)	BRACHIAL ARTERIAL PRESSURE		FEMORAL ARTERIAL PRESSURE		AVERAGE GLOMERULAR FILTRATION RATE (ML./MIN./1.73 M.²)	AVERAGE RENAL PLASMA FLOW RATE (ML./MIN./1.73 M.²)	AVERAGE RENAL BLOOD FLOW RATE (ML./MIN./1.73 M.²)	AVERAGE FILTRATION FRACTION (DECIMAL)
				PHASIC (mm. Hg)	MEAN* (mm. Hg)	PHASIC (mm. Hg)	MEAN* (mm. Hg)				
A. *Men*											
LED	28	1.83	48.8	155/75	100	105/85	90	127	533	1,043	0.238
B. *Women*											
McC	18	1.85	38.2	180/75	105	100/75	85	130	445	718	0.292
AHL	16	1.46	40.6	130/70	90	95/65	75	115	369	624	0.312
KEN	19	1.70	34.6	140/75	98	82/65	70	131	381	577	0.344
Average (women)	18	1.67	37.6	150/73	98	92/68	77	125	398	640	0.316
Standard deviation (women)	2	0.20	3.3	26/3	7	9/6	9	9	41	60	0.026

*Electrically integrated.

adequate size of these 3 groups (Table II). When our women without heart failure were compared with reported values for normotensive subjects (Fig. 1 and Tables I and IV),[18, 19] the results were similar to those found in our comparison of the corresponding groups of men.

TABLE IV. MEAN RENAL VASCULAR RESISTANCE IN SPECIAL GROUPS

CHARACTER OF GROUP	NUMBER IN GROUP	10^3 DYNES SEC. CM.$^{-5}$			
		RA*	R'E*	RV*	R*
Man with coaretation and incipient congestive heart failure	1	2.211	1.998	1.914	6.123
Women with coaretation in modest heart failure	3	1.934	3.268	3.148	8.326
Women with coaretation without heart failure	3	3.950	2.422	1.896	8.271
Normal women†	17	3.032	2.028	2.108	7.172
Standard deviation		±0.919	±0.358	±0.484	±1.675

*See footnotes to Table II for key to abbreviations.
†References 18 and 19.

In coaretation patients not in failure (Table I) upper compartment systemic arterial pressure was elevated in all phases, but the pulse pressure was wide. Conversely, a narrow pulse pressure in the lower arterial compartment was associated with a low systolic, somewhat elevated diastolic, and normal mean pressure level. This suggests that the hemodynamic changes in the upper arterial compartment may represent adaptive changes which serve to maintain ample perfusion pressure for abdominal viscera. These observations tend to confirm the view of Gupta and Wiggers[21] that the narrowed pulse pressure below the aortic constriction reflects physical damping of the arterial pressure pulse wave. Although 5 of 11 coaretation men had diastolic pressure of 90 mm. Hg or more in the lower compartment, the mean pressure of the group averaged only 94 mm. Hg and was almost identical with that of the normotensive subjects ($p = <0.8$).

Inspection of the renal hemodynamic findings reveals no striking deviation from normal volumes. The mean renal blood flow rate falls in the lower normal range, and the glomerular filtration in the upper normal range, with resultant elevation in the (derived) filtration fraction. Calculated renal vascular resistance values were normal in the preglomerular segment and elevated in the postglomerular components (net efferent and venular), in the presence of a normal or slightly elevated glomerular filtration pressure. This dynamic pattern might be explained in any one of three different ways: (a) that it is part of a generalized arteriolar constriction in both upper and lower systemic arterial compartments in response to a humoral influence, (b) that it is a localized manifestation of fright or apprehension in a group of patients who are younger than the normal controls, and (c) that it is an adaptive vascular reaction aimed at maintaining adequate glomerular filtration pressure in the face of a threatened fall in renal perfusion pressure. Evidence against generalized systemic arteriolar constriction has been presented in a previous communication.[9] Fright and apprehension seem unlikely as consistent factors in view of the demonstrated

hepatic hyperemia and the absence of tachycardia and hyperventilation in most of these patients under test conditions, although such factors may have been responsible for some of the changes observed in individual patients. Therefore, we favor the probability of a local autonomous renal vascular adaptive response to the chronic circulatory handicap. Even the presence of a normal renal arterial perfusion pressure may not be adequate for normal glomerular filtration in the absence of the usual pulsatile character of the blood flow through the arteriolar capillaries of the glomerular tufts, since in these coarctation patients the arterial stream is characterized by greatly damped pressure pulse waves of remarkably small amplitude. Reactive postglomerular vasoconstriction is a customary autonomous renal response to a fall in systemic arterial pressure, as in hypovolemic shock or in congestive heart failure. Examination of the renal vascular resistance values in the 3 coarctation patients in mild or moderate heart failure (Table IV) shows that, in the face of a reduction in renal perfusion pressure (Table III), the postglomerular resistance manifests a rather sharp rise.

A provocative question raised by these data is whether the locus of origin of the principal visceral circulatory demand stimulus resides in the kidneys or in their associated regional vasculature, since the renal circulation approaches normal while the circulatory rate to the other abdominal viscera is of a hyperemic order.[9]

There remains a question whether varying width of the arterial pulse pressure in the upper compartment may reflect the presence or absence of general arteriolar constriction in the lower arterial compartment.[5] Even in our patients who had elevated diastolic and mean brachial arterial pressure levels, we failed to find elevated mean femoral arterial pressure or significant reduction in renal blood flow rate. Therefore, we believe that differences in brachial arterial pressure patterns reflect differences in degree of development of collateral arterial circuits, rather than representing degree of arteriolar constriction below the aortic obstruction. We agree with Genest and associates,[5] however, that coarctation patients manifesting both systolic and diastolic hypertension in the brachial artery outnumber both those with only systolic hypertension and the uncommon individuals with normal blood pressure who are not in congestive heart failure (Tables I and III).

We chose to compare the coarctation of the aorta group with the group of patients having essential hypertension (Table II), since it is probable that renal hemodynamics in patients with hypertension of the idiopathic diastolic variety are the same as those in patients with hypertension secondary to demonstrated renal ischemia. Although the differences were not so striking in most instances as between the essential hypertensive and the normotensive groups, the renal hemodynamic characteristics of the coarctation group were significantly nearer normal than those of the essential hypertensive group, even in the face of a greatly lowered mean renal arterial pressure. This comparison demonstrates that the increased resistance in the kidney in patients with coarctation of the

aorta is located in a different segment of the renal vascular circuit and is of a different degree of magnitude than the renal resistance of the hypertensive group. Although this does not rule out the possibility that there is a humoral* stimulus for the modest increase in postglomerular resistance values in the coaretation of the aorta patients, it does render unlikely the possibility that the same type of stimulus which exists in patients with essential (and presumably renal ischemic) arterial hypertension is operative in patients with coaretation of the aorta.

When patients with coaretation of the aorta suffer congestive myocardial decompensation, they quickly lose the effectiveness of their previous hemodynamic adaptation. In them we find a diminution in both stroke volume and minute volume cardiac output, a fall in brachial arterial pressure, and a reduction in both femoral arterial pressure and renal blood flow. There is a correspondingly sharp rise in filtration fraction and in postglomerular renal vascular resistance values. This special group of coaretation patients may have caused confusion in those previous reports in which general hemodynamic observations were not made in order to segregate them from compensated patients with coaretation of the aorta. This probably has created a false impression of the incidence of diminished renal circulation in this condition.

Our interpretation of the data presented in this paper is that the renal circulation in uncomplicated patients with coaretation of the aorta is essentially normal. We believe that the observed minor changes in blood flow and vascular resistance represent a local renal regulatory response either (a) to the narrowed pulse amplitude or (b) to a threatened fall in renal perfusion pressure associated with the congenital circulatory handicap. We have no evidence to support the view that there is generalized systemic arteriolar constriction in our patients with coaretation of the aorta. The subnormal total arterial resistance is especially striking in the splanchnic arterial bed but is found also in the upper arterial compartment in the majority of the definitely compensated patients.[9] This is in sharp contrast to the situation in experimental coaretation, where the evidence suggests that generalized arteriolar constriction occurs[23] and that a humoral vasoconstrictor may circulate.[24] It is likely that the difference between the clinical disorder and the experimental preparation is related to the difference in age of the subjects at the time of production of the aortic constriction and the opportunity of the human to develop adequate collateral arterial channels.

A point of great interest to us in support of the hypothesis that these patients do not have generalized systemic arteriolar constriction is their clinical course when untreated. They tend to die prematurely of congestive heart failure, rupture of small saccular aneurysms, or of dissection of the aorta—all lesions secondary to the abnormal stress produced by an excessive cardiac stroke volume or long-standing hypertension, or to the effects of these factors or other congenital defects.[15] They do *not* die of progressive renal disease, nor do they have a high incidence[26] of toxemia of pregnancy (actually the incidence of

*Epinephrine may produce this type of renal hemodynamic pattern.[23]

toxemia seems to be remarkably low, suggesting that some feature of the circulatory adjustment to the coaretation may exert a protective effect; e.g., the demonstrated hepatic hyperemia or the probably adequate placental circulation). They do not develop severe retinopathy with papilledema, exudates, and hemorrhages.[27, 28] Thus, they fail to exhibit several serious renal and vascular complications that one expects to encounter often in patients having essential arterial hypertension or the hypertension associated with primary renal disease.[29, 30]

Because of these physiologic and clinical observations we do not believe that the upper compartment hypertension frequently seen in these patients is secondary to renal ischemia. Failure of the blood pressure of coaretation patients to return to normal soon after surgical resection and anastomosis has been proposed[24, 31] as evidence for a renal mechanism in the production of hypertension. The long-term postoperative behavior of the arterial pressure is under study in our laboratory, but it should be stated that at least 3 other factors can and probably do contribute to the arterial hypertension seen after plastic repair of the aortic defect: (a) In patients with aortic coaretation the larger vessels of the upper compartment become atherosclerotic with advancing age,[32] probably as a result of injury by the chronic systolic hypertension there. Consequently, the postoperative brachial arterial pressure readings cited may be ascribed partly to inelasticity of these vessels and the proportionately reduced effectiveness of the aorta as a truly elastic reservoir and a secondary pump. Thus, these patients may have functionally "old" arteries above the aortic constriction, with significant degrees of atherosclerosis to account for the postoperative persistence of some systolic hypertension. (b) There is a tendency for the distal portion of the aorta below the constriction to be hypoplastic.[33] The resection of the coaretated segment may fail to convert the repaired aorta into a compression chamber of normal capacity, with resultant "physiologic foreshortening" of the aorta intermediate between the anatomic foreshortening prior to operation, on the one hand, and a systemic arterial tree of normal capacity, on the other. (c) The huge bed of collateral arteries developed by a compensated patient with coaretation of the aorta through a period of years must continue to contain and conduct significant amounts of blood for a long time after excision of the coaretated segment and anastomosis of the divided aorta. These greatly dilated and tortuous collateral arteries (e.g., the intercostals) cannot be expected to shrink promptly after operation to a normal size; consequently, total arterial resistance to outflow from the upper compartment remains subnormal. Moreover, an adjustment period of unknown duration must be required for the lower compartment systemic arterioles to develop the constrictor capacity needed to provide ample resistance to the marked postoperative increase in systolic pressure.[34] Meanwhile, in order to maintain adequate mean arterial pressure for the abdominal viscera and lower extremities, stroke volume cardiac output and upper compartment systolic arterial pressure must continue to be elevated until the *total* systemic arterial resistance rises to

normal. (The effective demand on the heart during this transitional postoperative period is the same as that seen in fever or in thyrotoxicosis or in the presence of a large systemic arteriovenous communication.) If the entire systemic arterial bed becomes sufficiently smaller and normally vasoconstrictive, all hemodynamic values may return to normal. Yet they can be expected to change only in proportion to the gradual reduction in the caliber of the collateral arteries, which may fail to shrink ultimately all the way to normal size, and to the development of normal arteriolar constrictive power in the lower compartment.

SUMMARY AND CONCLUSIONS

1. Measurements of renal blood flow and glomerular filtration rates, as well as brachial and femoral arterial pressure levels, were made in 21 patients (14 men and 7 women) with coaretation of the aorta. Segmental renal vascular resistance values were calculated in 18 of them. These data were compared with similar information obtained in our laboratory from 25 normotensive men and from 29 men with idiopathic systemic arterial hypertension. Because 1 man and 3 women had varying degrees of congestive heart failure when studied, they were excluded from these comparisons.

2. No important renal hemodynamic abnormalities were found in patients with coaretation of the aorta who were not in congestive heart failure. Mean arterial perfusion pressure in the lower systemic arterial compartment was not elevated in these patients. As judged by effective renal blood flow rates measured by the plasma clearance method, there was no evidence of renal ischemia.

3. In general the renal hemodynamic differences between our patients with coaretation of the aorta and normotensive subjects were small and can be explained as representing autonomous local renal vascular adaptive responses operating to maintain adequate glomerular filtration pressure despite a long-standing cardiovascular handicap. By sharp contrast, the coaretation pattern differed very significantly both qualitatively and quantitatively from that of patients with essential hypertension.

4. These observations support the hypothesis that generalized systemic arteriolar constriction is not characteristic of patients with coaretation of the aorta who are free of congestive heart failure, and that the upper compartment systemic arterial hypertension is not caused by a circulating humoral vasoconstrictor like that postulated for primary renal hypertension in man or for experimental coaretation of the aorta in laboratory animals.

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FOLIC ACID AND VITAMIN B_{12} IN PERNICIOUS ANEMIA

Studies on Patients Treated With These Substances Over a Ten-Year Period

J. J. Will, M.D., J. F. Mueller, M.D., C. Brodine, M.D., C. E. Kiely, M.D., B. Friedman, M.D., V. R. Hawkins, R.N., J. Dutra, M.D., and R. W. Vilter, M.D.
Cincinnati, Ohio

VITAMIN B_{12},[1, 2] folic acid,[3, 4] and folinic acid[5, 6] induce excellent hematologic responses in patients with pernicious anemia in relapse. The remissions thus induced usually cannot be maintained by folic acid or related substances unless vitamin B_{12} is given also.[7-10] Other cellular components, such as the pyrimidine bases, thymine,[11] uracil,[12] and orotic acid,[13] when given in very large doses, induce less dramatic hematologic recovery in these patients, and ascorbic acid[14] occasionally has this effect. Of all these compounds, only vitamin B_{12} has been demonstrated to protect the nervous system and to prevent hematologic relapse.[15, 16] Numerous observations in human beings and a vast literature in the biologic and biochemical sciences support the hypothesis of a chemical interrelationship between these substances.[17-19] Over the past 12 years, our Laboratory Staff has observed clinical and biochemical effects of these substances in patients with pernicious anemia. This report will describe our clinical experiences with folic acid and vitamin B_{12} given singly to such patients as maintenance therapy for as long as remissions continued, and several biochemical studies which indicate that vitamin B_{12} plays a role in the metabolism of folic acid. No evidence has been found that folic acid has a direct effect on vitamin B_{12} metabolism or increases the requirements of the pernicious anemia patient for this vitamin.

MATERIAL AND METHODS

Between 1945 and 1948, 46 persons with pernicious anemia were placed on a regime of folic acid, 30 mg. 3 times a week, given orally. Thirty-six of these patients have been followed for 1 to 10 years until hematologic or neurologic relapse occurred, until death or other circumstances removed them from the series, or until they were studied in a manner that involved the administration of vitamin B_{12}. Twenty-nine of these patients previously had been maintained satisfactorily on refined liver extract. Seven had had no treatment prior to the administration of sufficient folic acid to induce hematologic remission.

From the Department of Medicine, University of Cincinnati College of Medicine, and the Cincinnati General Hospital, Cincinnati, Ohio.

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Those patients who attended the outpatient clinic intermittently or died before the expiration of one year have been eliminated from the series.

This group of patients, maintained on folic acid, will be compared with two groups of patients with pernicious anemia maintained on vitamin B_{12} given parenterally for 1½ to 7 years. The first group of 19 patients received 20 γ of vitamin B_{12} at monthly intervals and the second group of 21 patients received 10 γ of vitamin B_{12} every two weeks.

Erythrocyte counts, hemoglobin levels, and the volume of packed red blood cells have been determined every 3 months or oftener by standard techniques. Counts of circulating reticulocytes and platelets were performed by the wet technique using Dameshek's stain. The fact that relapse had occurred was confirmed by examination of bone marrow films stained with the Wright-Giemsa stain. Neurologic examinations have been performed every 3 months or oftener if the patient offered a complaint ascribable to progressive neurologic degeneration. A hematologic relapse has been defined as a fall in erythrocyte count of 500,000 cells or more, a decline in hemoglobin of 1½ Gm. or more, and a reduction of the packed cell volume of at least 5 per cent. Neurologic relapse was established by finding one or more of the following signs: a progressive diminution of vibration perception in the ankles, knees, or fingers; the development of ataxia; positive Romberg sign; glove and stocking hypesthesia; and mental abberations subsequently relieved by the administration of vitamin B_{12}. If the appearance of clear-cut evidence of hematologic or neurologic relapse was not separated by more than two months, a combined relapse is recorded.

From these groups of patients, certain ones were selected to study the effects of (1) large doses of folic acid on the therapeutic activity in neurologic disease of minimal doses of vitamin B_{12}, (2) vitamin B_{12} on folic acid and folinic acid excretion in the urine, and (3) folic acid on vitamin B_{12} levels in the serum. The patients selected and the procedures which were carried out are described below.

Three patients who developed hematologic and neurologic relapses while receiving folic acid were given 50 mg. of folic acid orally each day and 7 to 10 γ of vitamin B_{12} parenterally at weekly intervals. Hematologic and neurologic responses were studied over a period of 4 to 7 months to determine whether the large doses of folic acid interfered with the effect of minimal doses of vitamin B_{12}.

Two patients who received folic acid for 10 years and one who received the vitamin for 5 years remained in excellent health. They had no evidence of hematologic or neurologic relapse and were studied in the following manner. Each patient was admitted to the hospital and given 10 mg. of folic acid orally each day. Plasma ascorbic acid[20, 21] and blood glutathione[22] levels were determined. Vitamin B_{12} levels in the serum were estimated using the *Lactobacillus leichmannii* method,[23] and folic and folinic acid levels were determined in 24-hour urine specimens daily for 3 days by the *Streptococcus faecalis*[24] and *Leuconostoc citrovorum*[25] methods, respectively. After this period of control, vitamin B_{12}, 1,000 γ, was given intramuscularly, and the tests were repeated each 24 hours for the next 3 days. Since these patients had been maintained on folic acid for a period of 5 to 10 years, the levels of folic and folinic acids in their urine specimens were high and, therefore, easily measurable. Furthermore, although their vitamin B_{12} stores were depleted, these patients were in hematologic and neurologic equilibrium. Therefore, they were deemed to be excellent test cases to determine whether the administration of vitamin B_{12} influenced the excretion of folic or folinic acids. A change in the excretion of these substances may be construed to indicate a change in folic acid metabolism. After the study was completed, vitamin B_{12} labeled with Co^{60} was administered by mouth and the amount of radioactivity which appeared in the urine in 24 hours was determined by the method described by Schilling.[26] The test was repeated after a suitable interval with labeled vitamin B_{12} and intrinsic factor.* This was done to confirm the original diagnosis of

*Intrinsic factor was generously supplied by Organon, Inc., Orange, N. J.

pernicious anemia. There was little question as to accuracy of the diagnosis since two of these patients had had well-documented megaloblastic anemia and posterolateral column degeneration responsive to liver extract on their first admission to the hospital.

Serum vitamin B_{12} levels were determined two or more times in a randomly selected group of patients with pernicious anemia that had been controlled for 2 to 7 years by the parenteral administration of 10 γ of vitamin B_{12} every two weeks or 20 γ every month. These samples were analyzed for comparison with the vitamin B_{12} levels of patients treated a similar length of time with folic acid and to determine whether these small doses of vitamin B_{12} were sufficient to maintain serum vitamin B_{12} levels in a normal range, as well as to maintain those patients in a state of hematologic and neurologic remission.

Vitamin B_{12} serum levels were determined in 3 of these patients for 3 months before and 7 to 12 months after 5 mg. of folic acid daily was added to the regime. In 2 of these 3 patients, 10 mg. of folic acid daily was administered for an additional 5 to 8 months. In each of these cases, the average serum vitamin B_{12} level was above 100 γγ per milliliter.

In 3 additional patients in whom serum vitamin B_{12} levels were below 100 γγ per milliliter and in 2 patients with serum vitamin B_{12} levels above 100 γγ per milliliter, vitamin B_{12} dosage was increased to 30 γ every 2 weeks and finally to 60 γ every two weeks. After the serum vitamin B_{12} levels had stabilized above 100 γγ per milliliter, folic acid was added in doses of 10 mg. per day and the serum levels of vitamin B_{12} were redetermined over a period of 3 months. These experiments were performed to determine whether the administration of folic acid altered the amount of vitamin B_{12} in the serum.

The reliability of our assay for vitamin B_{12} was checked by sending specimens of plasma on these patients to another laboratory for assay by the *Lactobacillus leichmannii* method. A comparison was made also with vitamin B_{12} levels determined in these patients by the *Euglena gracilis* method.* Levels determined by the *Euglena gracilis* method were somewhat different, although in the same range (Table I).

TABLE I. SERUM VITAMIN B_{12} LEVELS ASSAYED BY THE LACTOBACILLUS LEICHMANNII AND EUGLENA GRACILIS METHODS

PATIENT	L. LEICHMANNII ASSAY γγ/ML. (UNIV. OF CINCINNATI)	L. LEICHMANNII ASSAY γγ/ML. (TULANE UNIV.)	E. GRACILIS ASSAY γγ/ML. (UNIV. OF LOUISVILLE)
A. M.	90	120	140
D. H.	120	110	150
N. C.	130	80	280
R. B.	130	110	200
J. C.	170	200	250

RESULTS

The Effects of Long-Term Folic Acid Therapy.—The 36 patients who received folic acid as maintenance therapy reacted in the following ways (Fig. 1). Within 6 months one patient had had a hematologic relapse, one a neurologic relapse, and one a combined hematologic and neurologic relapse. At the end of a year, 4 more had had neurologic relapses, and 3 combined neurologic and hematologic relapses. By the end of the second year, 2 more patients had had the combined type of relapse. In the third year, there were 5 hematologic relapses; in the fourth year, 2 hematologic, one neurologic, and two combined relapses. In the fifth year there were no relapses, but in the sixth year there was one neurologic relapse and one combined hematologic and neurologic relapse. In the seventh year there was one hematologic relapse. Those patients who developed hematologic relapse alone were treated

*The authors wish to thank Dr. Grace Goldsmith, Tulane University, and Dr. Marion Beard, University of Louisville, for arranging these comparative assays.

with increased doses of folic acid up to 50 or 100 mg. daily. One or two hematologic remissions and relapses usually occurred within the next 3 months after each increase in the dose of folic acid. Eventually neurologic relapse occurred in each case treated in this fashion. Because of this procedure, 21 patients eventually developed neurologic relapse. Bone marrow aspirated from the sternum at the time of the second or third hematologic relapse, when the neurologic degenerative disease necessitated therapy with vitamin B_{12}, showed severe hypoplasia with only slight megaloblastic maturation defect in the few remaining cells. This effect has been described in greater detail in a paper published previously.[12]

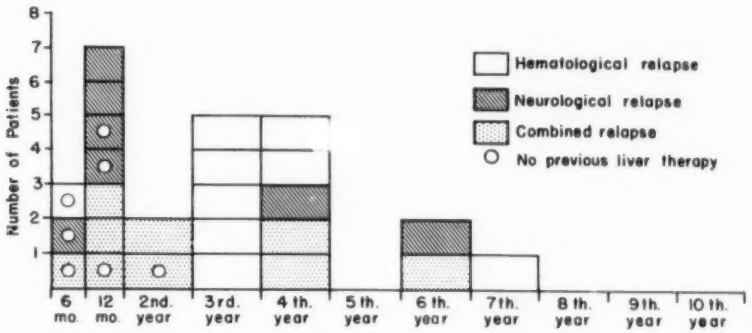

Fig. 1.—The number of hematologic, neurologic, and combined relapses which occured over a 10-year period in patients with pernicious anemia who were taking only folic acid (36 cases).

Nine patients developed glossitis just before or at the same time as the hematologic relapse. This lesion waxed and waned until adequate therapy was instituted.

TABLE II. PATIENTS WHO DID NOT RELAPSE WHILE BEING TREATED WITH FOLIC ACID

INITIALS	YEARS ON FOLIC ACID	REASON FOR TERMINATION OF TREATMENT
1. L. H.	4	Disappeared
2. C. S.	5	Vitamin B_{12} test
3. A. H.	3	Died, myocardial infaret
4. M. H.	3	Manic-depressive psychosis
5. A. P.	10	Vitamin B_{12} test
6. C. S.	10	Vitamin B_{12} test
7. N. T.	3	Died, gastric carcinoma
8. L. W.	1½	Died, gastric carcinoma
9. E. W.	1½	Died, cause unknown
10. M. S.	6	Moved away; on vitamin B_{12}
11. M. F.	2	Died, gastric carcinoma

Those patients who were well controlled on refined liver extract injections before the folic acid experiment was begun tended to relapse much later than those who were treated with folic acid from the time that the diagnosis was made. The latter group relapsed within the first 2 years of the folic acid experiment (Fig. 1).

TABLE III. VITAMIN B_{12} 10 γ INTRAMUSCULARLY EVERY 2 WEEKS

PATIENT	SEX	DURATION OF THERAPY (YEARS)	AVERAGE VITAMIN B_{12} SERUM LEVEL AT END OF STUDY ($\gamma\gamma$/ML.)	HEMATOLOGIC DATA (END OF THERAPEUTIC PERIOD)					
				R.B.C. (MILLIONS)	HB (GM.)	PACKED CELL VOLUME (PER CENT)	MCV (CU. μ)	MCH ($\mu\mu$/CELL)	MCHC (PER CENT)
F. R.	M	7	75	3.81	11.4	34	89	29	33
A. M.	M	6	120	4.52	16.0	45	99	35	35
L. J.	F	6	40	4.15	14.5	40	96	34	36
N. S.	F	5	200	4.44	14.9	40	90	31	37
E. M.	F	5		3.79	12.3	36	94	32	34
R. H.	M	5	150	4.28	13.8	44	102	32	31
A. K.	F	4½		4.50	15.0	44	97	33	34
C. H.	F	4½	90	4.20	12.6	40	95	30	31
N. G.	F	4	120	4.20	14.6	40	95	34	36
S. B.	F	4	150	4.28	14.8	41	93	33	35
A. W.	M	3		4.84	16.2	48	99	33	33
R. B.	F	3	100	4.11	14.0	41	99	34	34
E. E.	F	3		4.20	15.5	43	102	32	31
D. E.	M	3	135	3.80	13.5	39	102	35	34
G. Y.	M	2½	50	4.26	14.5	43	100	34	33
C. H.	M	2½	90	5.50	18.8	52	94	34	36
M. M.	F	2		4.50	14.8	46	102	32	32
S. P.	F	2		3.61	11.6	37	102	32	31
J. B.	M	2	150	4.50	15.3	46	102	34	33
J. C.	F	1½	50	4.51	15.3	44	97	33	34
L. P.	F	1½		4.70	17.0	51	108	36	33

Eleven patients did not relapse; 8 of these were lost from the series because of death or some other circumstance unrelated to the folic acid treatment (Table II). The remaining 3 patients were continued on folic acid for 5, 10, and 10 years, respectively, without evidence of hematologic, neurologic, or glossal relapse. Finally studies were performed which involved the administration of vitamin B_{12} and will be described later.

The Effect of Small Amounts of Vitamin B_{12} on Neurologic Lesions.—Three patients with neurologic relapse principally of the peripheral neuritis type, and which had developed while they were taking folic acid, were given 7 to 10 γ of vitamin B_{12} intramuscularly at weekly intervals. Folic acid dosage, which had been increased from 30 mg. 3 times weekly to 50 mg. daily, was continued at this level. Neurologic symptoms and signs gradually receded over a period of 11 to 12 weeks, normal erythrocyte levels were attained in 9 to 10 weeks, and, although the regime was continued for 4 to 7 months, no evidence of relapse developed. Ultimately the folic acid was discontinued (see Case Reports). All the other patients with neurologic relapse were continued on 50 or 100 mg. of folic acid daily and received 5 γ vitamin B_{12} daily or every other day by parenteral injection. Many of the signs of neurologic dysfunction were severe. All patients who had neurologic signs for less than 3 weeks made a full recovery of function, although, in several, the ability to perceive the tuning fork in the lower extremities did not return. Patients who had symptoms and signs for a longer period recovered function more slowly and less completely. It was never necessary, however, to discontinue folic acid to facilitate recovery.

Metabolic Studies on Patients on Folic Acid for 5 to 10 Years.—Two patients (C. S. and A. P.) who had received no other medication than folic acid, 30 mg. 3 times weekly or 10 mg. daily for 10 years, and one patient (E. S.) who had received a similar regime for 5 years without relapse were selected for study. Serum vitamin B_{12} levels per milliliter were 30, 15, and 10 $\gamma\gamma$, respectively. These levels are below the limits of accuracy of the assay procedure and can be considered to be 0. Ascorbic acid plasma levels were 1.37 and 1.13 mg. per cent and glutathione blood levels were 309 and 280 μM per 100 ml. cells in C. S. and A. P. These determinations were not performed in E. S. At the end of the experiment, tests with vitamin B_{12} labeled with Co^{60}, given orally according to Schilling's procedure, demonstrated that these patients behaved in a fashion identical with our other pernicious anemia patients. They excreted in the urine 0.6, 0.1, and 0.0 per cent of ingested radioactivity in the 24-hour period after the injection of 1 mg. of untagged vitamin B_{12}. Later, when the labeled vitamin B_{12} was given with intrinsic factor, C. S. and A. P. excreted 7.6 and 9.0 per cent, respectively. E. S. did not return for this test.

Each patient was maintained on 10 mg. folic acid daily. After the vitamin B_{12}, ascorbic acid, and glutathione levels were obtained and before the Schilling procedure was performed, each patient was given 1 mg. of vitamin B_{12} parenterally. The amount of folic acid and folinic acid excreted for the

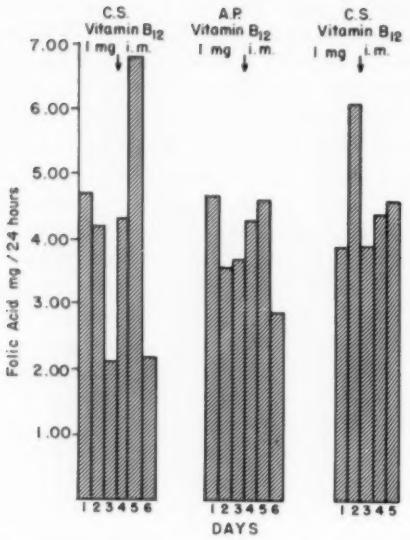

Fig. 2.—Vitamin B_{12} administration had no effect on the folic acid excretion in 3 persons with pernicious anemia who were depleted of vitamin B_{12} and saturated with folic acid.

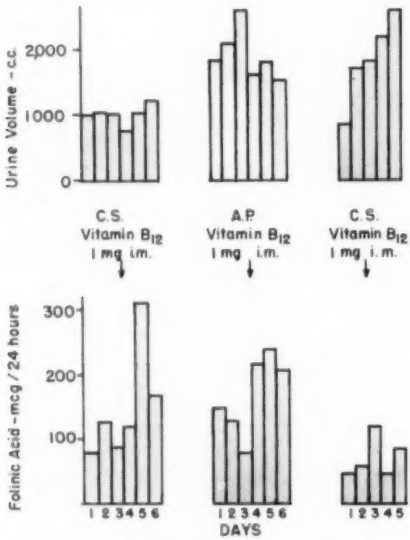

Fig. 3.—Effect of vitamin B_{12} administration on the folinic acid content of the urine of 3 patients with pernicious anemia who were depleted of vitamin B_{12} and saturated with folic acid.

3 days preceding the vitamin B_{12} dose and for the 3 days following it, are shown in Figs. 2 and 3. The output of folic acid did not change significantly. The output of folinic acid did, however, double. No significant change in the hematologic or neurologic examinations occurred, nor was a change expected, since there was no evidence of relapse prior to the vitamin B_{12} injection. One patient reported that he could maintain his balance better.

The Effectiveness of Small Doses of Vitamin B_{12} Administered Parenterally on the Maintenance of Hematologic and Neurologic Remissions in a Patient With Pernicious Anemia.—Tables III and IV indicate the hematologic effect of vitamin B_{12} given parenterally in doses of 20 γ monthly or 10 γ every two weeks as maintenance therapy for pernicious anemia. Neither hematologic, neurologic, nor glossal relapses occurred, although in 6 cases these schedules have been maintained for 6 to 7 years. These are very small doses, averaging 0.7 γ daily. Levels of vitamin B_{12} in the serum of a representative group of these patients will be found in Tables III and IV also. Most of the levels are low, many below 100 $\gamma\gamma$ per milliliter. The normal range in our laboratory is 250 to 700 $\gamma\gamma$ per milliliter. However, none of these patients had developed any symptoms or signs of ill health referable to these low levels. Several patients whose erythrocyte counts appeared to be low were given larger doses of vitamin B_{12} at the end of the experiment for periods up to 6 months. This procedure did not improve their hematologic status and vitamin B_{12} serum levels increased very slowly.

In 3 of these patients serum vitamin B_{12} levels were not changed by administration of folic acid 5 or 10 mg. daily for 12 months or longer. In 5 additional patients vitamin B_{12} serum levels increased slowly and to only a minor degree when vitamin B_{12} dosage was increased from 10 to 30 γ and then 60 γ every 2 weeks. However, in these patients, too, no additional changes were caused by the administration of folic acid (Table V).

DISCUSSION

Many investigators have demonstrated clearly that folic acid is not an acceptable therapeutic agent for the management of patients with pernicious anemia,[7-10, 27, 28] whereas vitamin B_{12} is entirely effective[29-31] even though the doses are so small that vitamin B_{12} blood levels remain low as demonstrated in this report. On the basis of the fulminating neurologic relapses that have been observed in patients maintained on folic acid, the opinion has been voiced that folic acid increases the need for vitamin B_{12} and, therefore, is dangerous. Our 10-year study corroborates the evidence that neurologic relapses occur much more frequently in patients maintained on folic acid than in those from whom vitamin B_{12} or liver extract is withdrawn.[32-34] The repeated hematologic remission and relapses that occurred in patients who received increasingly larger amounts of folic acid each time there was a relapse indicates that under these experimental conditions folic acid may deplete the body of vitamin B_{12} to the point of bone marrow exhaustion. However, this depletion of vitamin B_{12} apparently occurs because bone marrow activity and vitamin B_{12}

TABLE IV. VITAMIN B_{12} 20 γ INTRAMUSCULARLY AT MONTHLY INTERVALS

PATIENT	SEX	DURATION OF THERAPY (YEARS)	AVERAGE VITAMIN B_{12} SERUM LEVEL AT END OF STUDY (γγ/ML.)	HEMATOLOGIC DATA (END OF THERAPEUTIC PERIOD)					
				R.B.C. (MILLIONS)	HB (GM.)	PACKED CELL VOLUME (PER CENT)	MCV (CU. μ)	MCH (μμ/CELL)	MCHC (PER CENT)
E. W.	M	7	60	4.65	15.4	43	94	34	34
R. P.	F	6	80	4.90	16.4	46	93	33	35
J. B.	F	6		4.18	13.8	40	95	33	34
N. C.	F	5½		4.61	15.4	46	99	33	33
L. N.	F	5	60	4.60	15.8	48	104	34	32
J. S.	M	5	65	5.51	17.0	50	90	30	34
A. McN.	F	5	90	5.30	16.7	46	88	32	36
M. McA.	F	4½	20	4.74	16.2	44	92	34	37
D. H.	M	4½	150	4.51	16.2	45	99	35	36
I. H.	F	4	150	3.79	13.9	39	102	36	35
C. G.	F	4		4.70	15.8	44	93	33	35
A. F.	F	3½		5.30	17.2	49	92	32	35
I. O.	F	3		4.20	14.9	44	104	35	33
L. H.	F	2½		4.80	15.0	44	91	31	34
R. E.	F	2	195	4.51	14.5	44	97	32	32
F. B.	F	2		5.00	15.8	48	96	31	32
F. H.	M	2		5.30	16.9	48	90	31	35
D. S.	F	2		4.21	13.3	39	93	31	34
M. B.	F	1½		3.91	14.0	40	102	35	35

TABLE V. THE EFFECT OF FOLIC ACID ADMINISTRATION ON SERUM VITAMIN B_{12} LEVELS (γγ/ML.)

THERAPY	VITAMIN B_{12} 10 γ EVERY 2 WEEKS																							
	FOLIC ACID 5 MG. DAILY															FOLIC ACID 10 MG. DAILY								
MONTHS OF THERAPY	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Patient																							
A. M.	180	150	190	130	250	195	170	160	140	190	150	—	—	140	90	240	160	330	180	180	—	—	—
D. H.	—	150	—	—	—	—	—	120	210	250	250	230	180	—	—	140	110	140	—	150	—	—	210
S. E.	200	170	280	290	310	210	270	90	350	280													

THERAPY	VITAMIN B_{12} 10 γ EVERY 2 WEEKS			VITAMIN B_{12} 30 γ EVERY 2 WEEKS												VITAMIN B_{12} 60 γ EVERY 2 WEEKS						FOLIC ACID 10 MG. DAILY VITAMIN B_{12} 60 γ EVERY 2 WEEKS		
MONTHS OF THERAPY	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Patient																									
F. R.	60	70	60	130	145	100	90	170	—	—	—	—	160	130	—	—	150	220	—	250	—	340	280	290	300
J. C.	70	—	90	190	245	170	180	290	240	330	—	—	—	—	270	260	—	280	290	230	230	310	260	240	390
R. B.	50	—	170	130	130	190	135	100	230	165	—	—	—	—	150	130	240	260	—	—	—	230	250	340	300
R. H.	210	—	—	390	280	320	250	225	145	170	280	210	—	—	180	230	280	330	250	240	360	350	455	470	320
N. G.	180	190	170	140	190	145	220	220	260	—	—	—	—	310	230	—	—	350	270	240	370	210	430	430	410

utilization are maintained at a high level by folic acid. Folic acid apparently side-tracks from the nervous system to the marrow what little vitamin B_{12} is available to the pernicious anemia patient. Ultimately this very small supply is almost completely exhausted, and bone marrow hypoplasia and neurologic degeneration result. We have not, however, been able to find any evidence that excess folic acid increases the normal vitamin B_{12} requirements of the body. Less than 1 γ of vitamin B_{12} a day will alleviate peripheral neuritis in the face of large doses of folic acid; 2 to 5 γ a day will do the same thing in much more fulminating cases of cord degeneration. That folic acid does not invariably result in neurologic degeneration is attested by the observation that patients who previously have had neurologic degenerative disease may remain in remission on folic acid for as long as 10 years, although their vitamin B_{12} levels at the end of this period are as low as those usually found in patients with pernicious anemia in relapse. Obviously, these patients absorbed some vitamin B_{12} from the diet but in such small amounts that neither the Schilling test nor vitamin B_{12} blood levels indicated any difference from other patients with pernicious anemia who relapsed early in the course of the experiment or from the average patient with pernicious anemia in relapse. The fact remains that these minute and unmeasurable amounts of vitamin B_{12} were sufficient to prevent the appearance of symptoms and signs of deficiency. Perhaps the high reduced ascorbic acid levels found in the serum of these patients increased the effectiveness of the available vitamin B_{12}.

Our data do not support the theory that folic acid has a direct effect on vitamin B_{12} metabolism. They suggest only that minimal amounts of vitamin B_{12} may become completely inadequate when the chemical reactions of the cells in which both vitamin B_{12} and folic acid are intimately involved are temporarily stimulated by the administration of excess folic acid.* Fortuitous circumstances could explain data which have been proposed as evidence that folic acid lowers high vitamin B_{12} blood levels in patients with pernicious anemia treated with both substances.[35] Our experiments do not show such an effect. On the other hand, our results support the large body of evidence that vitamin B_{12} has an effect on folic acid metabolism.

About 50 per cent of patients with pernicious anemia in relapse have subnormal folic acid levels in the blood.[36] After a dose of vitamin B_{12}, serum[36] and urine[12] folic acid levels fall, but after a day or two rise again. Patients with nutritional macrocytic anemia given vitamin B_{12} excrete more than the usual amount of folic acid after several days.[37] These observations have been interpreted to mean that immediately available folic acid is used rapidly in hematopoiesis stimulated by the vitamin B_{12} injection, and that, later, the vitamin B_{12} mobilizes folic and folinic acids from tissue stores.

Persons with pernicious anemia, basically deficient in vitamin B_{12}, respond hematologically to folic acid, although this is but a temporary effect. If folic acid functions as a coenzyme in blood formation, this observation implies

*Recent observations reported by Bok and associates support this hypothesis (J. LAB. & CLIN. MED. 51: 667-671, 1958).

that lack of vitamin B_{12} has caused a deficiency of the active folic acid catalyst, probably N^{10}-formyl tetrahydrofolic acid. Furthermore, persons with pernicious anemia in relapse, given a test dose of 1 to 5 mg. of folic acid, excrete less folic acid and much less folinic acid than do normal persons or pernicious anemia subjects who are in remission.[38] This observation has been interpreted to indicate that tissue depots retain folic acid and, to an even greater degree, folinic acid when vitamin B_{12} is deficient, and that these are released when vitamin B_{12} is given. An alternate hypothesis is that vitamin B_{12} is necessary for the conversion of folic acid to folinic acid. In support of this, Girdwood[39] found more folic acid than folinic acid in the liver of a patient with pernicious anemia in relapse whereas in the liver of a normal person there is usually a preponderance of folinic acid. Our observations, that vitamin B_{12} increases the folinic acid levels in the urine of patients with pernicious anemia maintained in remission for many years by folic acid, provide additional evidence supporting such interrelationships. Since these patients showed no evidence of hematologic or neurologic abnormality, yet were severely depleted in vitamin B_{12}, any change induced by the administration of this vitamin would not be likely to result from a rapid increase in cell division. On the other hand, since the urine of the patients contained much larger amounts of folic and folinic acids than the normal person, the problem of measuring a change in the output of these substances was much simpler than in persons with normal or low levels in the urine. When vitamin B_{12} was given to these subjects, folinic acid excretion increased in the urine. Folic acid output did not change. It is unlikely that this was simply the result of increased cellular activity, utilization of folic acid metabolites, and increased urinary excretion of end products. As mentioned before, the patients were selected to avoid this contingency and they did not show any objective clinical change after the vitamin B_{12} was given. The data could be explained also as a direct effect of vitamin B_{12} on the conversion of folic acid to folinic acid, or on the release of folinic acid from its tissue conjugates, or on some other biochemical reaction essential for the formation of N^{10}-formyl tetrahydrofolic acid.

Folinic acid is no more effective than folic acid in patients with hematologic relapse of pernicious anemia.[5, 40] Therefore, it is unlikely that catalysis of this step by vitamin B_{12} is a critical process, although it may be the one we have studied in these experiments. Bethell and his associates[41, 42] showed that folic acid conjugate was not easily split by patients with pernicious anemia in relapse until after liver extract was given. He postulated an effect of vitamin B_{12} on the folic acid conjugate system. The same conjugate system releases folinic acid from its conjugates. Therefore, this system may be the one responsible for the increased output of folinic acid following the administration of vitamin B_{12}.

Nichols and Welch[43, 44] have recorded observations of a 4-year-old child with pernicious anemia which indicate a relationship of vitamin B_{12} to an unknown stage of folic acid metabolism. During a hematologic relapse which occurred while this child was receiving vitamin B_{12}, folic acid, and ascorbic

acid orally, an unidentified microbiologically active reduced metabolite of folic acid was found in the urine. After vitamin B_{12} was administered parenterally, this metabolite disappeared and was replaced by normal folic acid metabolites including folinic acid.

All of these observations, including our own, suggest the hypothesis that normal folic acid metabolism is dependent on an adequate supply of vitamin B_{12}.

These observations and the hypothesis derived from them do not imply that vitamin B_{12} functions in hematopoiesis only through folic acid metabolism. There is ample evidence from studies with bacteria and experimental animals that vitamin B_{12} has an independent effect on nucleotide formation, but the exact mechanism is unknown. Only one of its many functions involves it directly in folic acid metabolism but, through this function, it probably influences the transfer of single carbon units.

SUMMARY AND CONCLUSIONS

1. Vitamin B_{12}, given parenterally in a dose averaging 0.7 γ per day, has maintained persons with pernicious anemia in good health for 6 to 7 years. No deleterious effect has been found which can be ascribed to subnormal vitamin B_{12} serum levels characteristic of this group of patients.

2. Only 3 of 36 patients with pernicious anemia were maintained for 10 years in a satisfactory hematologic and neurologic condition on folic acid therapy alone. The great majority of these patients relapsed in the first 4 years. Those who had had no previous liver extract or vitamin B_{12} therapy relapsed sooner than those previously treated with these preparations. Those patients who did not relapse had, nevertheless, extremely low serum vitamin B_{12} levels.

3. Neurologic relapse occurred much more frequently in the group treated with folic acid than in patients reported by others who had been deprived of liver extract or vitamin B_{12}. However, only minute amounts of vitamin B_{12} were necessary to reverse these neurologic changes. No evidence has been found that folic acid changes the requirements of human beings for small amounts of vitamin B_{12} or influences vitamin B_{12} metabolism in a direct way.

4. Evidence is presented which suggests that vitamin B_{12} influences folic acid metabolism by releasing folinic acid from its tissue conjugates, by facilitating the reduction of folic acid to folinic acid, or by promoting the formation of the N^{10}-formyl derivative of reduced folic acid (N^{10}-formyl tetrahydrofolic acid).

CASE REPORTS

CASE 1.—D. S., aged 52, was admitted Feb. 26, 1948, with 800,000 erythrocytes per cubic millimeter and 3 Gm. per cent of hemoglobin. Her bone marrow was megaloblastic, but her neurologic system was normal. She was treated with an experimental liver extract over a period of 10 days with minimal response, and then was given 20 units of refined liver extract over a 10-day period with good reticulocyte response and erythrocyte rise. Folic acid, 30 mg. 3 times a week, was begun on April 15, 1948. By July, 1948, her hematologic data were normal. Erythrocytes number 4.31 million per cubic millimeter,

hemoglobin was 13.6 Gm. per cent, and the hematocrit was 40 per cent. In July, 1949, she complained of aching and fatigue in her legs, she was unstable on Romberg test, the ankle jerks were absent and vibration was barely perceived on the knees and ankles. Blood counts were still normal. By September she complained of numbness and tingling sensation in the hands and feet, vibration perception had diminished further, and her erythrocyte count had fallen to 3.6 million cells per cubic millimeter, the hemoglobin to 12.8 Gm. per cent. Folic acid was increased to 50 mg. daily. This therapy resulted in a temporary increase in erythrocytes and hemoglobin, but the signs and symptoms of peripheral neuropathy progressed. The tongue became smooth and red along the edges. By Nov. 17, 1949, vibration was no longer perceived in the knees, the reflexes were absent in the lower extremities and there was a stocking type of hypesthesia to pin prick and light touch. Romberg test was positive. Folic acid, 50 mg. daily, was continued, and 10 γ vitamin B_{12} was given parenterally at weekly intervals. By Dec. 1, 1949, the tongue appeared normal, vibration was barely perceptible in the right leg, and she was no longer ataxic on Romberg test. Gradual improvement continued. By Jan. 12, 1950, paresthesias and stocking hypesthesia had disappeared. By Feb. 2, 1950, all deep reflexes were obtainable and vibration perception in ankles and knees was 50 per cent of normal. The patient had no complaints and her hematologic values had returned to normal. Folic acid and vitamin B_{12} were continued until July, 1950. At this time, since there was no evidence of hematologic or neurologic relapse, the folic acid was discontinued. The patient had averaged 1.4 γ of vitamin B_{12} daily during the period of neurologic recovery.

Case 2.—J. S., aged 57, had his first relapse of pernicious anemia in 1934 and had been treated successfully with liver extract, 20 units monthly, until October, 1945. At this time he was given folic acid, 30 mg. 3 times a week, instead of the liver extract. He had had severe spinal cord and peripheral nerve degenerative disease at the time of his first admission, but at the time that folic acid therapy was instituted, the only neurologic abnormalities were a reduction of vibration perception in the ankles and hypoactive ankle jerks. By May, 1947, he was in severe hematologic and mild neurologic relapse. Hematologic values returned to slightly subnormal levels when thymine was given, and, since no further change occurred in either hematologic or neurologic findings when 10 units of liver extract were given over a period of 10 days, the patient was given folic acid again. By September, 1949, glossitis was noted. This sign waxed and waned until November, 1950, when it became very severe. Folic acid dosage was increased to 50 mg. daily, but no improvement occurred in the glossal manifestation. By March 29, 1951, he had become very weak; his tongue was fiery red and smooth; vibration perception was absent over the knee, ankles, and right wrist; knee and ankle reflexes could not be obtained and Romberg test was positive. He walked with a broad base. There was no evidence, however, of hypesthesia to pin or light touch, and no evidence of lateral column involvement. He was given 7 γ vitamin B_{12} parenterally once a week and was continued on 50 mg. folic acid daily. At this time, his erythrocytes numbered 2.80 million cells per cubic millimeter, his hemoglobin 11.0 Gm. per cent, and the hematocrit 33 per cent. Bone marrow was hypocellular and showed only slight evidence of megaloblastic maturation arrest. By April 5, his tongue was no longer red or sore, and his appetite had returned. By May 10, 1951, Romberg sign was negative and ataxia no longer was evident. The erythrocytes and hemoglobin were normal. Vibration was appreciated in the right wrist on May 24 and in the knees on July 7. By this time, glossal papillae had regenerated. Folic acid was discontinued July 21, 1951, when the patient's neurologic and hematologic status had reached pre-folic acid conditions. Improvement had occurred on an average of 1 γ vitamin B_{12} per day in spite of large doses of folic acid.

Case 3.—E. M., a 46-year-old Negro woman, was found to have pernicious anemia in 1945 and responded well to refined liver extract, 15 units twice a month. In May, 1947, folic acid, 30 mg. orally given 3 times a week, was substituted for the liver injections. When this change was made, her erythrocyte count was 4.02 million cells, her hemoglobin

was 12.4 Gm. per cent and her hematocrit was 40 per cent. About 3 years later in July, 1950, she complained of soreness of the tongue, which was red at the tip and sides. Her erythrocytes numbered 3.41 million cells, her hemoglobin 12.5 Gm. per cent, and her hematocrit 37 per cent. The glossitis waxed and waned, and in December, 1950, she complained of numbness of the right foot. By this time her erythrocyte count was 2.9 million cells per cubic millimeter, her hemoglobin was 11.2 Gm. per cent, and her hematocrit was 34 per cent. The dose of folic acid was increased to 30 mm. daily; the glossitis and anemia improved temporarily, but the complaints of numbness of the feet increased. At this time, vibration perception was reduced in the ankles and knees. In February, 1951, she complained of numbness of the left hand and in March, 1951, hypesthesia of the hands and feet to pin prick and light touch was noted. The ankle jerks were hypoactive. Vibration was barely perceived in the ankles. Her right side was weak and she tended to fall to the right when she walked. Her erythrocyte count was 3.15 million cells per cubic millimeter, her hemoglobin was 12.8 Gm. per cent, and her hematocrit was 37 per cent. Sternal bone marrow contained bizarre metamyelocytes and band cells but the erythrocyte series appeared normal. Vitamin B_{12}, 7 γ intramuscularly, was given at weekly intervals and folic acid was continued as before. After 2 weeks the patient was worse. Ankle jerks were absent and Romberg sign was positive. All signs of glossitis had disappeared, however. The first evidence of neurologic improvement appeared on April 26 when she stated she felt better. By May 3, ankle jerks were obtained and the hypesthetic areas on the hands and feet had disappeared. The Romberg test was negative and the patient could walk without falling. By June 1, vibration perception was 30 per cent of normal in the knees. Her erythrocyte count was 3.9 million cells per cubic millimeter, hemoglobin 12.9 Gm. per cent, and hematocrit 39 per cent. Slow improvement continued until Oct. 16, 1951, when folic acid was discontinued.

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THE DISTRIBUTION OF LIPID AND PHOSPHOLIPID IN PAPER ELECTROPHORESIS OF THE SERUM LIPOPROTEINS IN NORMAL SUBJECTS AND IN PATIENTS WITH ATHEROSCLEROSIS

M. A. Chapin, Ph.D., M.D., and Samuel Proger, M.D., F.A.C.P.
Lewiston, Maine, and Boston, Mass.

IN SUPPORT of the view that atherosclerosis may be a metabolic disease, numerous investigators have found an association with increased beta lipoproteins of serum,[1-4] with increases in the cholesterol content of the beta lipoproteins,[5, 6] with increases in the low-density lipoproteins,[7] and with an increased serum cholesterol-phospholipid ratio.[8-10] Most of the evidence indicates atherosclerosis to be associated with abnormal increases in the beta lipoproteins. Changes in the alpha lipoproteins are slight and consist of slight decreases in the lipid and cholesterol.[3, 5]

While cholesterol, as a more insoluble component of the lipids, has been considered to play a dominant role in the metabolic disturbance of lipids promoting atherogenesis,[11] its status as a single causal entity has frequently been questioned, perhaps mainly because in any series of patients with coronary disease there are many with normal concentrations of cholesterol in the serum. Increasing attention has therefore been given to the other lipids[12] of serum and to the lipid-protein relationships[3] in terms of possible lipid stabilizing effects.

Recently, for the same reason, the phospholipids have also come under scrutiny.[13-16] The constancy of the cholesterol-phospholipid ratio has been emphasized,[17] and it has been shown that no less than a 1:1 molar ratio of phospholipid to free cholesterol exists in the plasma at all times.[16] In experimental canine atherosclerosis, Davidson and associates[13] have found phospholipid to increase at the average rate of but one mole for every five moles of cholesterol. A consequence of such findings has been the growth of a concept that the available phospholipid determines the amount of cholesterol which can be kept in solution in the plasma and that relative increases of cholesterol may result in instability of the lipid solution.[18]

It, therefore, seemed desirable to investigate changes in the phospholipid content of the serum beta lipoproteins in normal subjects and in those with atherosclerosis with the hope that such comparison might give a better understanding of the nature of lipid disturbance in this disease. This study has been based on a simplified method[19] utilizing paper electrophoresis as a means of separation of the beta and alpha lipoproteins in multiple strips of paper, some of which

From the Department of Medicine, Central Maine General Hospital, Lewiston, Maine, and the New England Center Hospital, Boston, Mass.
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were stained directly for estimation of lipid distribution between the two groups, and the remainder stained for phospholipid after differential extraction and removal of the other lipid components. This technique has permitted a gross estimation of the beta and alpha lipid fractions, of the beta and alpha phospholipid in these fractions, and indirectly, the amount of phospholipid in the total lipid of serum.

MATERIALS AND METHODS

All chemical determinations were done on blood serum obtained in the fasting state, 14 to 16 hours after a light evening meal. Each serum was refrigerated at 5° C. and the various chemical and electrophoretic determinations accomplished within 24 to 48 hours. Several groups of persons were studied. These consisted of 20 young men and 22 young women, all presumably normal and healthy; 18 older persons, also normal by the usual examinations and routine tests; 20 subjects, of ages comparable to the older age group, who had had recent episodes of myocardial infarction and were 1 to 3 weeks in the recovery phase at the time of testing; 10 with uncomplicated diabetes mellitus; 3 with nephrosis; 5 with essential hypercholesterolemia; and 2 with myxedema.

Serum cholesterol and esters were determined by the method of Schoenheimer and Sperry.[20] The method of Fiske and Subbarow[21] was used for the determination of lipid phosphorus and the factor of 25 for its conversion to phospholipid. Triglycerides were calculated from the difference between the total fatty acids[22] and the sum of the estimated phospholipid and cholesterol ester fatty acids. Total serum lipids were calculated as the sum of the values for free cholesterol, estimated cholesterol esters, phospholipid, and triglycerides.

A previously described modification[19] of the technique of Kunkel and Slater[6] for paper electrophoresis of serum lipoproteins which permits the simultaneous use of multiple strips of Whatman No. 1 filter paper, was employed throughout this study. A 0.02 ml. sample of the serum being tested was applied to each of the strips in a similar manner and location. The entire procedure of electrophoresis, drying of strips, and subsequent staining of lipids was standardized and accomplished in the same way for each determination. Ten or more of the dried strips from each serum electrophoretic run were treated with boiling acetone saturated with magnesium chloride to extract all lipids other than the insoluble phospholipid. These strips, along with at least 6 other unextracted strips from the same serum run, were subjected to the same staining procedure with 1 per cent Sudan Black B in 50 per cent ethanol (triple filtered) in one large tank. At the end of the 12-hour staining period, the strips were washed by successive immersion in 4 containers of 40 per cent ethanol for 15 minutes each. Since there was some fading of these dyed strips on exposure to bright light, all strips were dried and kept in the dark until manually scanned with a photoelectric densitometer using a 590 mμ filter. From the curves relating optical density and distance along the strip the areas corresponding to the beta and alpha albumin lipid zones were measured by planimetry and the percentage distribution of the lipids in the 2 lipoprotein zones calculated. With careful technique and attention to detail, the reproducibility of this method is within ± 5 per cent for total lipids and ± 9 per cent for phospholipid.

RESULTS

In view of the fact that the findings here reported indicate both the total lipid and phospholipid distribution in the beta and alpha lipoproteins and therefore apply only to the lipid moiety of these fractions, the presentation of results by application of usual terminology becomes complicated and cumbersome. In the following discussion it has been found advantageous to refer to the total lipid in the beta and alpha lipoproteins as the beta and alpha lipids, and to the phospholipid in each fraction as the beta and alpha phospholipid.

Distribution of Total Lipid and Phospholipid.—

A. *Young men and women*: The young women of this group were from 19 to 30 years old and the young men were 16 to 32 years old. There was no family history of diabetes or hypercholesterolemia in any of the subjects. The data on these two groups are listed in Table I. The average values for the group of men for serum total lipid and for total and free cholesterol were slightly greater and the values for phospholipid slightly lower than the corresponding values for women. In these young people, phospholipid represented 41 per cent of the serum lipids in young women and 34 per cent of those in young men.

TABLE I. DISTRIBUTION OF SERUM LIPID AND PHOSPHOLIPID IN YOUNG MEN AND WOMEN BETWEEN 16 AND 32 YEARS OLD

DETERMINATION	NORMAL WOMEN (22 SUBJECTS)		NORMAL MEN (20 SUBJECTS)	
	AVERAGE	RANGE	AVERAGE	RANGE
Chemical Findings (mg. per cent)				
Total cholesterol	152	90-228	158	102-191
Free cholesterol	37	12-79	43	25-56
Cholesterol esters	115	75-165	115	72-156
Lipid phosphorus	8.6	7.0-11.4	8.3	6.5-10.1
Phospholipid (factor 25)	215	170-285	208	163-253
C-P ratio	0.71		0.76	
Triglycerides	82	0-169	162	96-217
Total lipid	527	424-656	614	467-694
Phospholipid per cent of total lipid	41	33-46	34	27-39
Electrophoretic Findings (per cent)				
Total lipid distribution				
Beta zone	71	58-83	76	65-85
Alpha zone	29	17-44	24	15-39
Phospholipid distribution				
Beta zone	67	59-83	64	53-84
per cent of total lipid	31	18-43	26	15-40
per cent of beta lipid	44	26-64	34	26-47
Alpha zone	33	17-54	36	16-47
per cent of total lipid	15	5-25	14	8-20
per cent of alpha lipid	52	31-80	60	44-87
Phospholipid per cent of total lipid	43	31-53	40	32-49
Calculated Distributions				
Beta lipid (mg. %)	374	259-525	466	314-565
Beta phospholipid (mg. %)	144	98-188	133	87-212
Phospholipid per cent of beta lipid	39	28-53	29	20-38
Alpha lipid (mg. %)	154	85-258	148	92-239
Alpha phospholipid (mg. %)	71	40-154	75	41-110
Phospholipid per cent of alpha lipid	46	33-64	51	35-69
Beta-alpha lipid ratio	2.4	1.1-4.9	3.3	1.6-5.6
Beta-alpha phospholipid ratio	2.0	0.9-4.9	1.8	0.9-5.2

The sera of young women averaged more alpha lipids (154 mg. per cent) and less alpha phospholipid (71 mg. per cent) than was found in the sera of young men (148 and 75 mg. per cent, respectively). The sera of young men averaged 24 per cent greater beta lipids (466 mg. per cent, compared with 374 mg. per cent), and nearly 10 per cent less beta phospholipid (133 mg. per cent, compared with 144 mg. per cent) than was found in the sera of young women. These differences in the lipid and phospholipid distribution in the two main lipoprotein fractions of serum from young people of different sexes, are shown

in Fig. 1 where the values for the beta lipids are related to the serum total lipid of each individual examined, and in Fig. 2, where the values for the beta phospholipid are related to the serum phospholipid in each instance.

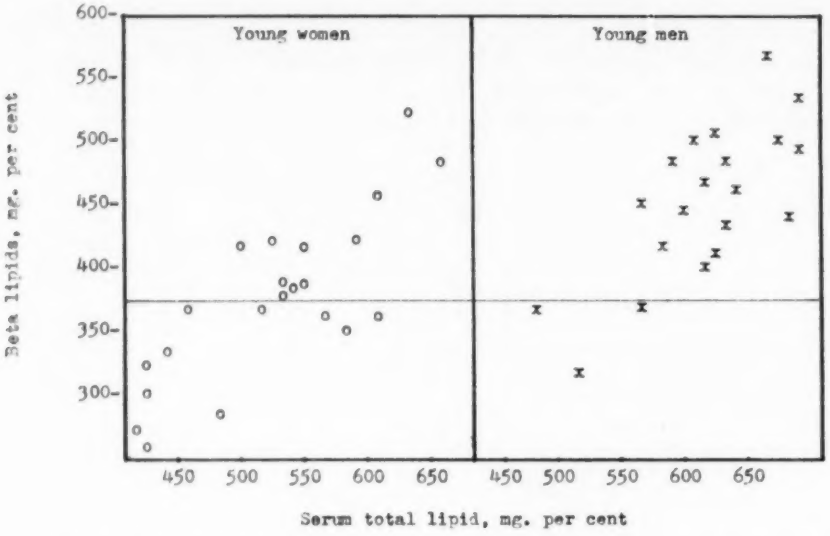

Fig. 1.—Comparison of the beta lipid values for sera of young women and men. The majority of young men have beta lipids in excess of the average for young women, represented by the horizontal line.

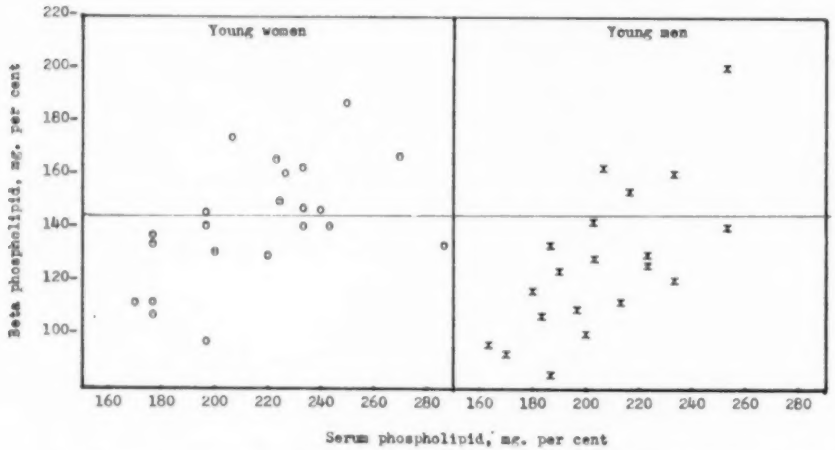

Fig. 2.—Comparison of the beta phospholipid values for sera of young women and men. The majority of young men have beta phospholipid values less than the average for young women, represented by the horizontal line.

These findings indicate the sera of young men to have greater beta lipids with less beta phospholipid, and less alpha lipids with more alpha phospholipid than is found in the sera of young women, with the greatest difference being evident in the beta lipoproteins.

B. *"Normal" older group:* In this group of 18 subjects, there were 10 postmenopausal women and 8 men, ranging in age from 40 to 66 years. The persons comprising this group had been hospitalized for routine examinations or had been admitted for functional complaints. None showed evidence of organic disease or overt atherosclerosis. The average findings for this group are listed in the second vertical column of Table II for comparison with the findings for young women, selected as the reference group.

Chemical analyses of the sera of this group showed an increase in total lipids and slight increases in cholesterol and phospholipid in comparison with the sera of young women. After electrophoretic separation, 82 per cent of the serum lipids migrated with the beta lipoproteins, compared to the 71 per cent distribution for the group of young women, and 73 per cent of the phospholipid accompanied the beta lipids. From calculated concentrations this group showed greater amounts of beta lipids and phospholipid than was found in the group of young women; the phospholipid percentage composition of the beta lipids was 32 per cent and comparatively lower. Both the alpha lipids and phospholipid were less in this group than in the group of young women.

Clinically normal older persons appear, therefore, to have increased beta lipids and phospholipid, with the former being disproportionately increased and the latter amounting to a difference of 7 per cent less than its composition of the beta lipids of young women. In reciprocal fashion, there were less alpha lipids and phospholipid than was found in the sera of young women. No sex difference in the distribution of these lipids was detected in this group of older people.

C. *Persons with known coronary atherosclerosis:* This group of 20 subjects, all men and 38 to 63 years old, represented instances of recovery from recent myocardial infarction. Three of the group were recovering from a second attack. Six were having intermittent angina pectoris. All patients with diabetes mellitus and those with suspected essential hypercholesterolemia were excluded from this group. As noted in Table II, the chemical analyses of the sera, when averaged, showed moderate increases in all of the lipid components as compared with those of young men and particularly of young women. Phospholipid content averaged 35 per cent of the total lipid, approximating the percentage composition of the sera of older persons and of young men, but notably less than the 41 per cent composition found in young women.

Electrophoretic separation of the serum lipids of this group disclosed a greater proportion (83 per cent) of the total lipid in the beta fraction than was found in the groups of normal subjects. Both the alpha lipids and alpha phospholipid values were decreased. The average beta lipids of 646 mg. per cent represents a 70 per cent increase over that for young women. Thus, while the beta phospholipid was somewhat increased, this increase was disproportionate,

TABLE II. DISTRIBUTION OF LIPID AND PHOSPHOLIPID IN YOUNG WOMEN, IN OLDER PERSONS, AND IN CONDITIONS ASSOCIATED WITH ATHEROSCLEROSIS

DETERMINATION	NORMAL YOUNG WOMEN (22 SUBJECTS)	NORMAL OLDER PERSONS (18 SUBJECTS)	RECENT MYOCARDIAL INFARCTS (20 SUBJECTS)	UNCOMPLICATED DIABETES MELLITUS (10 SUBJECTS)	NEPHROSIS (3 SUBJECTS)	ESSENTIAL HYPERCHOLESTEROLEMIA (5 SUBJECTS)	UNCOMPLICATED MYXEDEMA (2 SUBJECTS)
Chemical Findings (mg. per cent)							
Total cholesterol	152	155	225	213	336	438	378
Free cholesterol	37	47	63	64	97	141	119
Cholesterol esters	115	108	162	149	239	297	259
Lipid phosphorus	8.6	9.8	10.7	11.2	18.0	14.6	18.4
Phospholipid (factor 25)	215	245	268	280	450	367	460
C-P ratio	0.71	0.70	0.85	0.77	0.75	1.09	0.82
Triglycerides	82	208	187	172	706	203	748
Total lipid	527	681	770	752	1642	1210	1760
Phospholipid per cent of total lipid	41	36	35	27	27	30	26
Electrophoretic Findings (per cent)							
Total lipid distribution							
Beta zone	71	82	83	85	94	89	87
Alpha zone	29	18	17	15	6	11	13
Phospholipid distribution							
Beta zone	67	73	74	76	88	75	71
per cent of total lipid	31	31	30	35	21	23	18
per cent of beta lipid	44	38	36	35	22	26	21
Alpha zone	33	27	26	24	12	25	29
per cent of total lipid	15	12	10	10	3	6	7
per cent of alpha lipid	52	55	50	51	63	46	55
Phospholipid per cent of total lipid	43	43	39	39	24	28	25
Calculated Distributions							
Beta lipid (mg. %)	374	560	640	650	1560	1080	1530
Beta phospholipid (mg. %)	144	179	198	213	398	275	326
Phospholipid per cent of beta lipid	39	32	31	33	26	25	21
Alpha lipid (mg. %)	154	121	130	102	82	130	230
Alpha phospholipid (mg. %)	71	66	70	67	52	91	134
Phospholipid per cent of alpha lipid	46	55	54	66	68	70	58
Beta-alpha lipid ratio	2.4	4.6	4.9	6.4	19.0	8.3	6.7
Beta-alpha phospholipid ratio	2.0	2.7	3.5	3.1	7.7	3.0	2.4

All figures represent average determinations.

and its percentage composition of the beta lipids averaged 31 per cent. This was considerably less than was found in the group of young men and is conspicuous in view of the gross increases in the other beta lipid components. Triglycerides, which were also increased in the sera of this group, represented some portion of the latter, since it migrates in large part with the beta lipids.[23] The alpha lipids and phospholipid of this group were both decreased in actual average amounts below those of young women. Phospholipid appeared to make up somewhat more than half of the alpha lipids in these subjects.

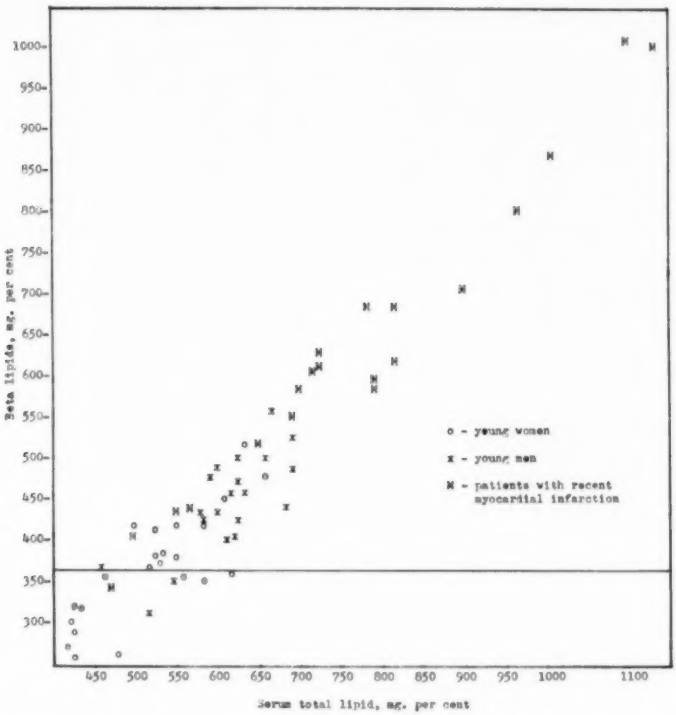

Fig. 3.—The relationship of beta lipids to serum total lipid in young women, young men, and in patients with coronary atherosclerosis. The horizontal line represents the average beta lipid concentration for young women. Values for the majority of young men and for all but one of the patients with recent myocardial infarction fall above this line.

These data, therefore, indicate the sera of patients with coronary atherosclerosis to have considerably increased beta and slightly decreased alpha lipids, giving a beta-alpha lipid ratio of 4.9, as compared to the ratio of 2.0 found in young women. The phospholipid distribution, however, followed the same pattern found in the sera of young men and of the older group of normal persons, i.e., while the beta phospholipid increased moderately as compared with the sera of young women, this increase was not in proportion to the great increase

in the beta lipids as a whole. Thus, the beta phospholipid of this group was 20 per cent less than it would be if it were increased in the same ratio as in young women.

Figs. 3 and 4 are scatter diagrams showing the relationship of beta lipids to serum lipids and of beta phospholipid to serum phospholipid for the individual members of this group as well as those of young men and women. The deranged content of the beta lipids with regard to phospholipid is thus emphasized.

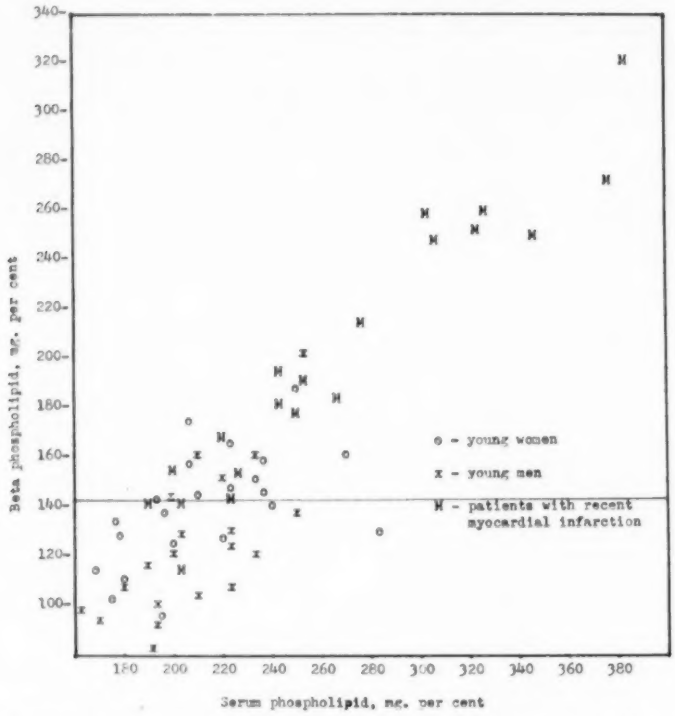

Fig. 4.—The relationship of serum and beta phospholipid in young men and women and in patients with coronary atherosclerosis. The horizontal line represents the average beta phospholipid concentration for the sera of young women. The values for most of the sera of young men fall below this line.

D. *Diseases with high incidence of atherosclerosis*: 1. *Diabetes mellitus*: Eight instances of this disease, uncomplicated by ketosis, infection, or overt coronary disease, comprised this group. By chemical analysis, all of the serum lipid components were increased over the normal, particularly the serum phospholipid which averaged higher than that of the preceding group with coronary atherosclerosis. As shown in the listing of results from this group inTable II, there were increased amounts of total lipid and phospholipid in the beta lipids of the sera examined, yet phospholipid represented but 33 per cent of this fraction.

The alpha lipids were decreased but phospholipid was present in nearly normal amount as compared with that for young women. Some decrease in the other alpha lipid components is thus indicated to be present in diabetes. The beta-alpha lipid ratio was nearly threefold that of young women.

2. *Nephrosis*: The 3 subjects selected for this group had typical nephrotic syndromes with varying degrees of edema, hypoalbuminemia, proteinuria, and hypercholesterolemia. The nephrosis in all subjects was believed to be secondary to chronic glomerulonephritis. The abnormal beta lipoproteins of this group of sera migrated with an increased mobility, often encroaching on the alpha lipid fraction and making exact separation and subsequent analysis frequently difficult by the method employed. The greatly increased lipid content of these sera was reflected in an increase in all components, including triglycerides. From the data listed in Table II, the greatest portion of this increased lipid is seen to be present in the beta lipoproteins which contained 94 per cent of the total lipid and 88 per cent of the phospholipid which was present in the serum. By actual calculation, however, phospholipid comprised but 26 per cent of the greatly increased beta lipids, or considerably less than that of any of the other groups studied. Reciprocally, the alpha lipids and phospholipids were decreased in actual amounts, with the latter appearing to make up 68 per cent of the alpha lipids of this group. One of these 3 subjects whose serum total lipid was 2,300 mg. per cent, appeared to have an alpha lipid consisting almost entirely of phospholipid. This unusual result, verified on repeated testing, while perhaps representing some peculiarity of the method of extraction or of staining as it applies to those abnormal lipoproteins, indicates the alpha cholesterol and triglyceride to be greatly decreased in nephrosis. Further studies are in progress with an aim to clarify this finding, particularly since similar results have been observed in some instances of acute infections hepatitis and obstructive jaundice, confirming the earlier observations of Kunkel and Slater.[6]

3. *Essential hypercholesterolemia*: The 5 subjects in this group, 3 men and 2 women, were from 32 to 54 years old. All had had angina pectoris or myocardial infarction. One had fairly extensive tendon deposits of xanthomatosis and 4 had xanthelasma. Another, a 32-year-old woman with severe angina pectoris which was present before and after an earlier episode of myocardial infarction, had a strong family history of probable hypercholesterolemia (3 siblings had died between the ages of 22 and 34 of acute heart disease), and has since died with a recurrent myocardial infarction. From chemical analyses of the sera, the markedly increased lipids reflected increases in cholesterol and its esters and, to a lesser extent, in the phospholipid. In comparison with the sera of nephrotic patients, there was much less phospholipid and triglycerides. While the beta lipids were some threefold increased over that for young women, the beta phospholipid was but twofold increased and comprised only 25 per cent of the beta lipids. Of equal interest was the finding that while the alpha lipids were slightly decreased below those of young women in terms of actual amounts present (130 mg. per cent), the alpha phospholipid (91 mg. per cent) was increased over that for young women and represented 70 per cent of the alpha

lipids. This increase in the alpha phospholipid was of about the same degree as previously noted in the sera of patients with diabetes and nephrosis. It is interesting that in this disease which is characterized by great increases in serum cholesterol, alpha cholesterol appears to be decreased.

4. *Hypothyroidism*: The 2 patients in this group were 48- and 56-year-old women. Their basal metabolic rates were minus 36 and 40 per cent, respectively. Each had the classical clinical picture of myxedema. The serum total lipid was increased to an extent nearly equal to the average found in nephrosis, the serum total cholesterol was greater than in the latter but less than that found in hypercholesterolemia, and there were increased triglycerides. One of these sera was lactescent. The serum phospholipid was increased in degree comparable to that of the nephrotic sera. In these 2 subjects, 87 per cent of the serum lipids were present in the beta lipoproteins, giving the latter a lipid content of 4 times that for young women. Only 71 per cent of the serum phospholipid migrated in this beta fraction, with the result that the beta lipid content of phospholipid was but 21 per cent. This was the lowest concentration for beta phospholipid found in any of the sera examined in this study. Of equal interest was the finding that the calculated alpha lipids in this disease is apparently increased some 50 per cent, and the alpha phospholipid nearly 90 per cent, over the values for normal young women. More than one-half of the alpha lipids appear to consist of phospholipid in myxedema. The beta-alpha lipid ratio was increased to about the same degree as that in diabetic sera. The sera of both patients, after 6 months' treatment with desiccated thyroid, showed more normal lipid distribution patterns of the type described for the older age group of persons.

Summary of Findings in Atherosclerosis.—Both these and other[5] results appear to indicate that the serum lipids in coronary atherosclerosis and in diseases with a strongly atherogenic tendency show distinct changes when they are examined in terms of their component distributions in the beta and alpha lipoproteins and when compared with a reference group of normal young women. The general pattern of these changes appears to be as follows:

1. The beta lipids, as a group, are increased greatly.
2. The beta cholesterol is increased moderately.[5]
3. The beta phospholipid is increased slightly.
4. The phospholipid percentage content of the beta lipids is decreased greatly.
5. The alpha lipids are decreased moderately (excepting myxedema).
6. The alpha cholesterol is decreased slightly.[5]
7. The alpha phospholipid is decreased slightly (excepting myxedema).
8. The phospholipid percentage content of the alpha lipids is increased slightly.
9. The beta-alpha lipid ratio is increased greatly.
10. The beta-alpha phospholipid ratio is increased moderately.

These changes are visualized in Fig. 5, which illustrates in terms of milligrams per cent concentration the actual amounts of total lipid and phospholipid

found in the beta and alpha lipoproteins in each of the different groups studied. The lower bars represent the alpha and the upper bars the beta lipids, the whole bar in each instance representing the total lipid of each fraction. The shaded portion of each bar represents the amounts of beta and alpha phospholipid, and the percentage composition of each fraction is numerically inserted. It is seen that even though there is an increase in the beta phospholipid, this increase is disproportionately less than the increase in the beta lipids, and that the latter, in atherosclerosis, contain relatively less phospholipid than is found in the sera of young women.

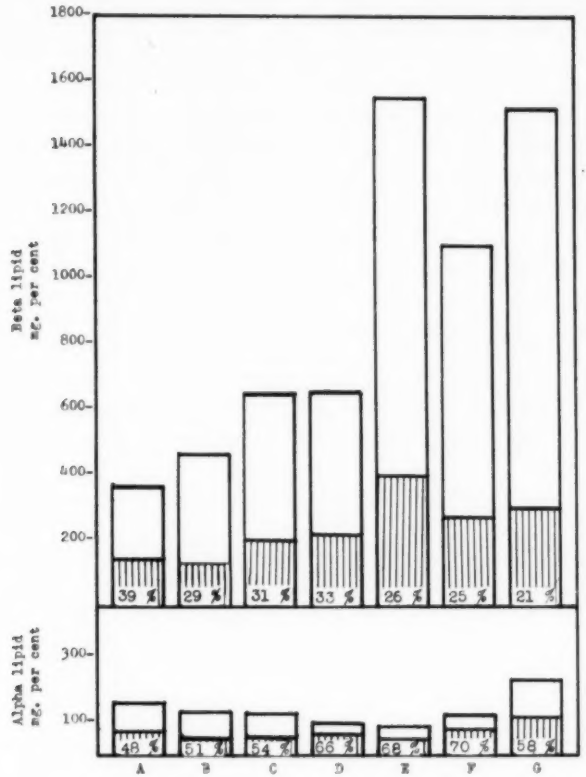

Fig. 5.—Relative lipid and phospholipid compositions of the beta and alpha lipoproteins in differing groups. A. Normal young women. B, Normal young men. C, Patients with recent myocardial infarction. D. Patients with uncomplicated diabetes. E, Nephrosis. F, Essential hypercholesterolemia. G, Myxedema. The portions of each bar representing phospholipid are striped and the percentage composition of each is numerically represented.

DISCUSSION

Additional information concerning the pattern of abnormal lipid distribution in atherosclerotic sera has appeared as a result of this study. If one accepts

the distribution of serum lipids and phospholipid of young women as the normal, or group of reference, then the sera of patients with coronary atherosclerosis have increased beta lipids which contain less than the normal ratio of phospholipid. A trend to this pattern was found in the sera of young men. Because of their potential importance, sex differences in the blood lipids have been sought by many investigators. Thus, Russ and associates[3] found young men to have more beta lipoprotein and less alpha lipoprotein than young women. Oliver and Boyd[5] found young men to have more beta cholesterol and less alpha cholesterol than young women. Nikkilä[1] and Fasoli,[2] by means of paper electrophoresis, had earlier demonstrated the same finding. Jones and co-workers[25] from a study of the low-density lipoproteins, found that 20 per cent of the men but only 2 per cent of the women between the ages of 31 and 40 have over 60 mg. per cent of the Sf 10-20 class of lipoproteins (believed to be of the beta fraction); a similar difference could not be demonstrated in older age groups. The data reported here indicate another sex difference involving the phospholipid composition of the beta lipoproteins, the latter, in young men, having more total lipid but relatively less phospholipid than those of young women.

This pattern is somewhat more distinct in the older age group of normal subjects of both sexes, the beta lipids being increased to a greater degree than the beta phospholipid. The trend is more distinct in the group of persons with coronary atherosclerosis and in other strongly atherogenic diseases. In these groups, the beta lipids are grossly increased whereas phospholipid constitutes a lesser percentage of this total. In all instances of patients with atherosclerosis, with the exception of myxedema, there are increases in the beta lipoproteins and decreases in the alpha lipoproteins of the serum. The significance of these reciprocal changes in the two lipoprotein fractions is unknown. Russ and coworkers[3] associated this finding with a diminished albumin content of the alpha fraction. The observations concerning the differences found in myxedema are unexplained.

The concept, revived by Boyd[26] and expressed by others,[8, 9, 13, 16] that phospholipids play an important role in maintaining stability of the plasma lipids and that lipid derangements resulting in decreased phospholipid concentration are associated with the development of atherosclerosis, provides a convenient interpretation of the findings of this study. It appears possible that this demonstration of a relative decrease in the beta phospholipid, occurring concomitantly with an increase in the other beta lipids—a disturbed distribution known to be present in atherosclerosis—signifies lessened stability in a system with a saturation limit.

It is known that the individual lipid fraction values, in the serum as well as in the two lipoprotein fractions, vary markedly from individual to individual, and that there is a wide variation in values obtained from normal persons. The variations in these values in sera from patients with atherosclerosis is even more marked. Mann[24] has emphasized that the variability (of lipid values) within a given sex and age group is very large, and that the measures of spread around

the mean are large, giving coefficients of variation of 30 to 50 per cent. Therefore, in this, as in any study of blood lipids, average values should be interpreted as trends and not as absolute limits. The statistical significance of the observed changes in the beta lipids and phospholipids in the groups of young men and women and in the group of patients with coronary atherosclerosis is indicated in Table III.

TABLE III

GROUP	SEX	NUMBER OF SUBJECTS	BETA LIPID (MG. PER CENT) Mean ± S.D.	p	BETA PHOSPHOLIPID (MG. PER CENT) Mean ± S.D.	p
Normal young women	F	22	374 ± 54.3		144 ± 22.8	
Normal young men	M	20	464 ± 42.8	<0.01	133 ± 27.1	<0.5
Patients with atherosclerosis	M-F	20	641 ± 137.9	<0.01	202 ± 62.0	<0.01

SUMMARY

1. A method, devised for the measurement of total lipids and phospholipids in the beta and alpha lipoproteins after electrophoretic separation of these serum fractions, has been applied to the sera of groups of normal young men and women, of older persons, and of patients with coronary atherosclerosis. Smaller groups of patients with diabetes mellitus, nephrosis, essential hypercholesterolemia, and myxedema have also been included. Cholesterol, phospholipid, triglycerides, and total lipids have been determined in the original serum and the total lipid and phospholipid in each of the two lipoprotein fractions.

2. The sera of young men have more total lipid with relatively less phospholipid in the beta lipoproteins, and less total lipid with relatively more phospholipid in the alpha lipoproteins than the sera of young women of comparable age.

3. The sera of patients with coronary atherosclerosis and of patients with atherogenic diseases have greatly increased total lipid with relatively less phospholipid in the beta lipoproteins, and markedly decreased total lipid with relatively more phospholipid in the alpha lipoproteins when compared with the sera of young women.

4. This pattern of abnormal lipid distribution in atherosclerosis has been shown to be most pronounced in the beta lipoproteins.

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THE DIFFERING MECHANISMS OF ACTION OF MERCURIALS, CARBONIC ANHYDRASE INHIBITORS, AND CHLOROTHIAZIDE AS DIURETIC AGENTS

RALPH V. FORD, M.D., AND J. B. ROCHELLE III, M.D.
HOUSTON, TEXAS

THE precise mechanism(s) of action of diuretic agents remains an enigma. Histochemical and in vitro chemical data indicate that perhaps mercurials and sulfonamides inhibit specific enzyme systems for the activation of the ion exchange mechanism for sodium reabsorption. In an effort to supplement these data with reference to renal diuretic mechanisms, this study has been performed. It was speculated that the responses to 3 groups of diuretics—mercurials (meralluride), carbonic anhydrase inhibitors (acetazoleamide), and chlorothiazide—in the urinary excretion of sodium might reveal indirect evidence concerning their mechanism of action.

This study was inspired by preliminary work in dogs by Pitts,[1] which suggested that mechanisms of certain diuretics were "competitive" while others were "noncompetitive." The further exploration of this matter in human beings comprises the substance of this report.

MATERIALS AND METHODS

The subjects of the studies were men with well-controlled chronic congestive heart failure due to hypertensive cardiovascular disease. At the time of the study the patients presented no detectable edema. They were maintained on the metabolic ward and fed a diet containing 50 mEq. of sodium and 3,000 ml. of distilled water per 24 hours. Their average 24-hour excretion of sodium was 45 mEq. in the control state. On the morning of the study, and until its completion, the patients were not fed. They drank 1,000 ml. of distilled water from 6 A.M. to 7 A.M. and 250 ml. every hour until the study was completed. Urine, collected in 30-minute periods throughout the study, was analyzed for sodium, potassium (analyzed by means of a Beckman flame photometer), chloride,[2] and bicarbonate.[3] Beginning at 7 A.M. and throughout the study isotonic sodium chloride solution was infused intravenously at a rate of approximately 650 μEq of sodium per minute. The purpose of this infusion was to prevent depletion of body stores of sodium which might be reflected in decreased urinary sodium during the latter parts of the study, especially after potent diuretic administration. Three diuretics, meralluride (Mercuhydrin), acetazoleamide (Diamox), and chlorothiazide (Diuril), were administered alone and in various combinations to determine the effects on the excretion of the various electrolytes. Contemporary observations of glomerular filtration rate (inulin clearance) and renal plasma flow (para-aminohippurate clearance) were made. Each segment of this study was repeated 5 times and the results are reported as the average values. In each value reported the mean effect is at least 3 times the standard deviation.

From the Departments of Medicine and Pharmacology, Baylor University College of Medicine, and the Veterans Administration Hospital, Houston, Texas.

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After four 30-minute control periods, a sustaining infusion of isotonic sodium chloride solution was administered at a rate of 650 μEq per minute and observations of the urinary electrolyte excretion were made for a total of 7 hours.

In order to determine the potency of the various diuretics in producing an increased sodium excretion, the next segment of the study was performed. The sustaining infusion of isotonic sodium chloride was started. After four 30-minute control periods the various drugs were given separately by intravenous injection, and the urinary electrolyte responses were observed in four 30-minute periods, or a total of 2 hours. These periods, selected after preliminary studies, revealed that maximum responses were seen in these periods. Although an occasional observation beyond this point revealed greater responses, this variation was not statistically significant. The effect of acetazoleamide on electrolyte excretion was observed at two dosages, 500 and 2,000 mg.; the dosages of meralluride were 20, 80, 160, and 320 mg. of mercury equivalent*; the dosages of chlorothiazide were 500, 2,000, and 4,000 mg.

In order to determine the possibility of competitive diuretic mechanisms other observations were made. The sustaining infusion of isotonic sodium chloride solution was started, and after 4 control periods meralluride was administered in a priming dose of 160 mg. mercury equivalent (4 ml.) and 160 mg. placed in the sustaining infusion. After a 2-hour period, during which time the excretion of sodium reached a maximum rate, a 500 mg. priming dose of acetazoleamide was given and the observations extended another 2 hours. Several days elapsed before the patient was used again as the subject of this study. However, since an ample quantity of sodium chloride had been provided during the study the body stores of sodium were only rarely depleted by the rather massive diuretic administration. Only rarely did a patient complain of muscular cramps, nausea, or diarrhea. In a subsequent study, the order was reversed. After a suitable control period, acetazoleamide was administered in a priming dose of 2,000 mg., and another 2,000 mg. was placed in the sustaining infusion of isotonic sodium chloride. After 2 hours, during which time the sodium response became maximal, meralluride (20 mg. mercury equivalent, 0.5 ml.) was administered intravenously as a priming dose and the observations continued as before. The purpose of these two studies was to observe the possible inhibition of sodium excretion by the second drug given after a maximum natriuretic effect had been obtained from the first drug.

Similar studies were done using meralluride 160 mg. (4 ml.) as a prime and 160 mg. as a sustaining infusion followed after 2 hours by 2,000 mg. chlorothiazide as a priming dose. Subsequently (on another day) chlorothiazide was given in a priming dose of 2,000 mg. (and 2,000 mg. sustaining) followed, after 2 hours, by a 80 mg. priming dose of meralluride (2 ml.).

Similar studies were performed using a priming dose of 2,000 mg. of acetazoleamide (and 2,000 mg. as sustainer) followed by 500 mg. chlorothiazide as a prime. On a subsequent day chlorothiazide was given first, 500 mg. prime and 500 mg. sustaining, followed by 2,000 mg. acetazoleamide as a prime.

A final study was performed using 160 mg. meralluride as prime (and 160 mg. sustaining), followed by 2,000 mg. acetazoleamide as prime (and 2,000 mg. sustaining), and both of these followed by a priming dose of 500 mg. chlorothiazide.

In each instance the dose of the first drug to be given was chosen as the one shown previously to produce a maximum sodium excretory response for that drug. The dose of the second drug was chosen as that one which was most nearly equal in potency (relative to sodium excretion) to the first drug. However, if the second drug evoked an increased sodium excretion, a dose one-half (or even one-fourth) the original dose was used to be certain that the factor of potency was not operative. On the other hand, if the second drug failed to evoke an increased sodium excretion, either the dose of the first drug was reduced by one-half (or one-fourth) or the dose of the second drug was doubled, whichever was more feasible.

*Forty milligrams mercury equivalent equals 1 ml.

RESULTS

A. The Response to Isotonic Saline Infusion (Fig. 1).—Under the conditions of this study the intravenous infusion of isotonic sodium chloride solution produced a threefold increase in the urinary excretion of sodium (and chloride) which became apparent within 2 hours. The rate of the infusion was approximately 650 μEq per minute of sodium and the excretory sodium rate was approximately 120 μEq per minute during the 7 hours of observation. Bicarbonate and potassium excretion was not altered. These patients, although previously edematous, presented no evidence of edema at the time of the study and had been on a moderately restricted salt intake for some time. During the remainder of the day and on the day following this procedure their sodium excretion was naturally greater than prior to the study as the kidney was able to excrete slowly the excess salt load presented by the infusion. These data furnish the background upon which is superimposed the effect of various diuretics under identical conditions in subsequent studies.

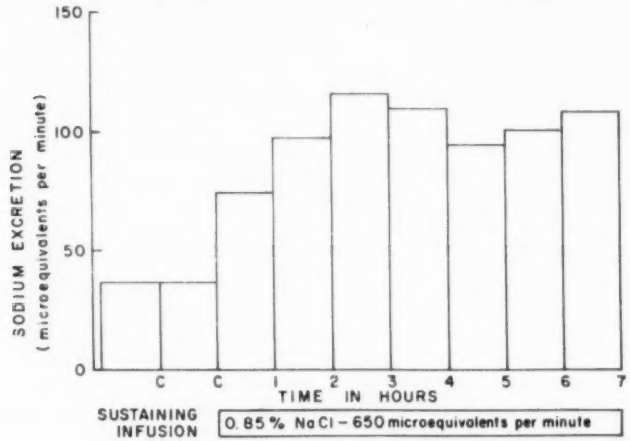

Fig. 1.—The response in sodium excretion to the infusion of isotonic saline in cardiac patients eating a diet containing 50 mEq. sodium daily. After two hours the sodium excretion is fairly stable during the continuous infusion of isotonic saline.

B. The Effect of Three Intravenously Administered Diuretics on the Excretion of Sodium and Other Electrolytes (Fig. 2).—During the infusion of isotonic sodium chloride, the intravenous administration of 500 mg. acetazoleamide produced a maximum sodium excretion of 300 ± 45 μEq per minute (control = 100 – 120) while a dose of 2,000 mg. produced a rate of 340 ± 52 μEq per minute illustrating no significant difference between these two dosages. The excretion rate of potassium was about 90 per cent that of sodium, bicarbonate excretion rate paralleled potassium, and the chloride excretion rate was depressed to less than the control value. The intravenous administration of meralluride produced a sodium excretion of 301 ± 52 μEq per minute after 20 mg. mercury equivalent (0.5 ml.), 610 ± 72 after 80 mg., 1,309 ± 120 after

160 mg., and 1,392 ± 137 after 320 mg. of meralluride. This illustrates a significant relationship of response in sodium excretion to the dose until the 160 mg. dose level is passed after which no additional response is observed. The excretion rates of potassium and bicarbonate were not altered; chloride excretion paralleled the sodium excretion. The intravenous administration of chlorothiazide produced a sodium excretion of 397 ± 36 μEq per minute after 500 mg., 815 ± 82 after 2,000 mg., and 892 ± 96 after 4,000 mg. of the drug.

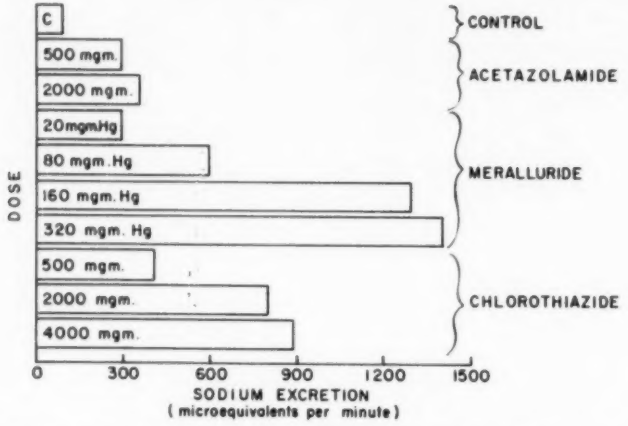

Fig. 2.—The effect of 3 diuretics given intravenously on sodium excretion. The comparative bar graphs illustrate a distinct dependence of sodium excretion upon the dose of meralluride and chlorothiazide but the last two doses show no significant differences.

This illustrates, as in the case of meralluride, that there is a significant relationship of sodium response to the dose of the drug until the 2,000 mg. dose is exceeded, beyond that further increase in dosage is not accompanied by an increased sodium excretory rate. The excretion rate of potassium and bicarbonate was increased to about 35 per cent of the sodium rate; chloride excretion paralleled the sodium excretion.

C. The Effect of Combinations of Meralluride and Acetazoleamide on the Excretion of Sodium and Other Electrolytes (Fig. 3).—After the administration of meralluride, 160 mg. mercury equivalent (4 ml.), as a priming dose and 160 mg. as a sustaining infusion, there was an increase in sodium excretion from a control value of 110 μEq per minute to 1,360 ± 170. As the sustaining infusion of saline containing the meralluride was continued and while the sodium excretory rate was maximum and stable (at the end of 2 hours) the intravenous administration of 500 mg. acetazoleamide, prime only, produced a further increase in sodium excretion to 1,920 ± 310 μEq per minute. Chloride excretion after meralluride paralleled sodium excretion while potassium and bicarbonate were not altered. After acetazoleamide was added, the chloride excretion rate was reduced to only 50 per cent of the sodium rate while bicarbonate and potassium excretion rates increased to 50 per cent of the sodium rate.

In a subsequent study the administration of acetazoleamide, 2,000 mg. prime and 2,000 mg. sustaining, produced an increase in sodium excretion to 345 ± 52 μEq per minute which was maximum and stable at the end of 2 hours. At this point a priming dose of meralluride 20 mg. (0.5 ml.) produced a further increase to 722 ± 119 μEq per minute. The excretion rate of bicarbonate and potassium after acetazoleamide was increased to about 90 per cent of the sodium rate while chloride excretion was depressed below the control rate. After meralluride was added, the chloride excretion rate increased to 80 per cent of the sodium rate while bicarbonate and potassium excretion rates were not altered from the original acetazoleamide effect.

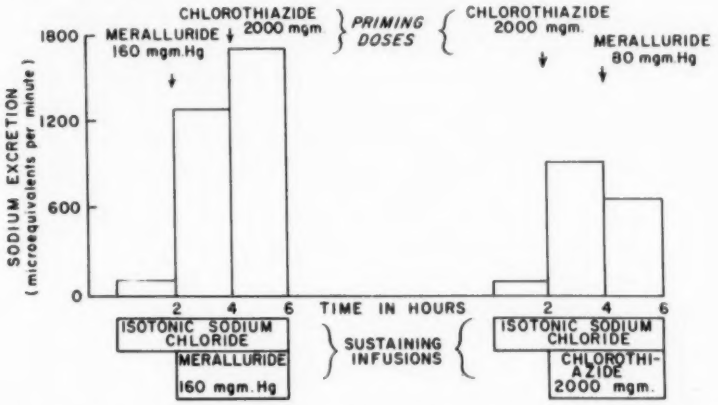

Fig. 3.—The effect of combinations of meralluride and acetazoleamide on sodium excretion illustrating noncompetitive mechanisms of action. Even with a maximum response from either meralluride or acetazoleamide, the administration of the other drug produces an additional increase in sodium excretion suggesting activation of a second "receptor site."

D. The Effect of Combinations of Meralluride and Chlorothiazide on the Excretion of Sodium and Other Electrolytes (Fig. 4).—After the administration of 160 mg. mercury equivalent of meralluride (4 ml.) as prime and 160 mg. as sustaining, the sodium excretion increased to 1,360 ± 170 μEq per minute which was maximum and stable at the end of 2 hours. At this point a priming dose of 2,000 mg. chlorothiazide produced a further increase in sodium excretion to 1,732 ± 158 μEq per minute. The chloride excretion rate was increased after meralluride to parallel the sodium rate while bicarbonate and potassium were not altered. After chlorothiazide was added, bicarbonate and potassium excretion rates were increased to about 35 per cent of the sodium rate while the chloride excretion rate was only 60 per cent of the sodium rate.

In a subsequent study the order was reversed so that a 2,000 mg. priming dose and a 2,000 mg. sustaining dose of chlorothiazide produced a sodium excretion of 860 ± 92 μEq per minute which was maximum and stable at the end of 2 hours. At this point a priming dose of meralluride, 80 mg. mercury equivalent (2 ml.), produced no further increase in the rate of sodium excretion (780 ± 82 μEq per minute). After the chlorothiazide administration the

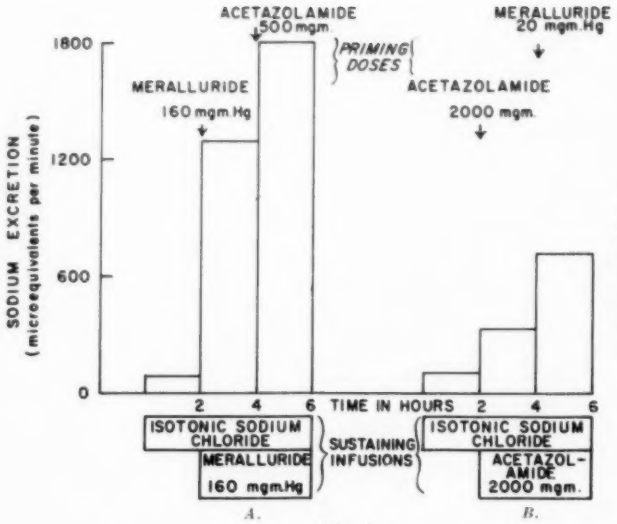

Fig. 4.

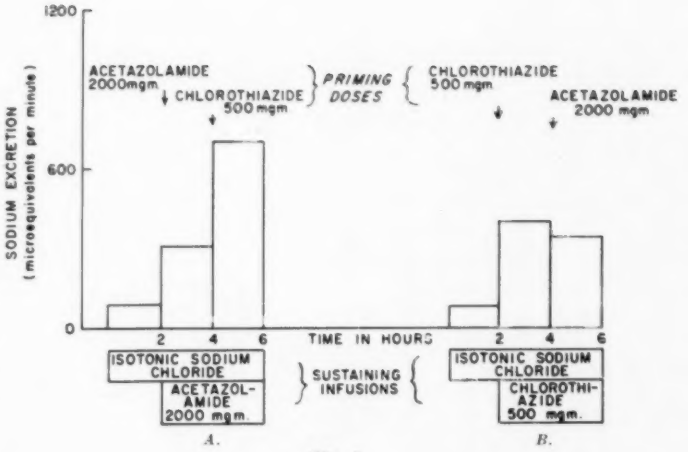

Fig. 5.

Fig. 4.—The effect of combinations of meralluride and chlorothiazide on sodium excretion illustrating noncompetitive (*A*) and competitive (*B*) mechanisms. The additional sodium excretion from chlorothiazide cannot be inhibited by meralluride but chlorothiazide can inhibit an additional sodium response from meralluride.

Fig. 5.—The effect of combinations of acetazoleamide and chlorothiazide on sodium excretion illustrating noncompetitive (*A*) and competitive (*B*) mechanisms. The additional sodium excretion from chlorothiazide cannot be inhibited by acetazoleamide, but chlorothiazide can inhibit an addition sodium response from acetazoleamide.

excretion rate of potassium and bicarbonate was increased to about 35 per cent of the sodium rate while chloride excretion paralleled the sodium excretion. When meralluride was added no significant changes in potassium, chloride, or bicarbonate were observed.

E. The Effect of Combinations of Acetazoleamide and Chlorothiazide on Electrolyte Excretion (Fig. 5).—The administration of 2,000 mg. of acetazoleamide as a prime and 2,000 mg. as a sustaining dose produced a sodium excretion of 345 ± 52 μEq per minute which was maximum and stable at the end of 2 hours. At this point, a 500 mg. priming dose of chlorothiazide produced an additional increase in sodium excretion to 720 ± 88 μEq per minute.

In a subsequent study the order was reversed so that 500 mg. of both a priming and a sustaining dose of chlorothiazide, produced a sodium excretion of 442 ± 65 μEq per minute which was maximum and stable at the end of 2 hours. At this point, a 2,000 mg. priming dose of acetazoleamide produced no further increase in sodium excretion (380 ± 51 μEq per minute).

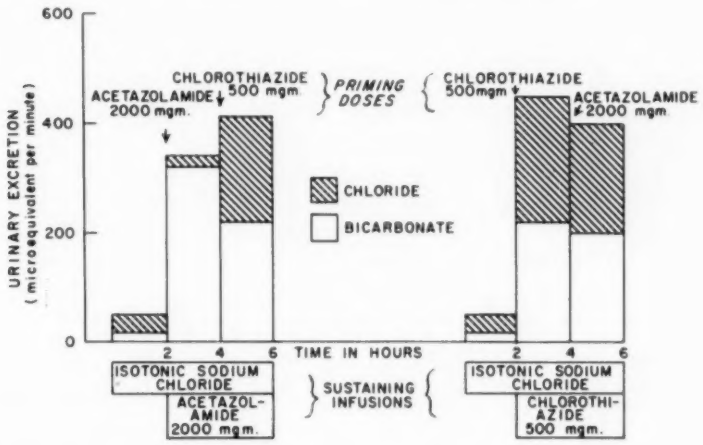

Fig. 6.—The effect of combinations of acetazoleamide and chlorothiazide on chloride and bicarbonate excretion. Chlorothiazide decreases the bicarbonate response produced by acetazoleamide but augments chloride.

To further elaborate on this situation a single typical study of the excretion of bicarbonate and chloride under the same conditions is presented in Fig. 6. After the priming and sustaining doses of acetazoleamide, bicarbonate excretion rate increases to 320 while the chloride excretion rate is 20 μEq per minute (control bicarbonate was less than 10 and chloride less than 40). After chlorothiazide was added as a prime (in the same study) bicarbonate excretion dropped to 220 and chloride increased to 190 μEq per minute. In a subsequent study, when chlorothiazide was administered first, bicarbonate excretion increased to 220 and chloride to 230 μEq per minute. However,

after acetazoleamide was added as a prime (in the same study) bicarbonate excretion rate was then 200 and chloride 200 μEq per minute. Potassium excretion (not graphed), in general, paralleled the bicarbonate excretion.

F. The Effect of Combinations of Meralluride, Acetazoleamide, and Chlorothiazide on the Excretion of Sodium and Other Electrolytes (Fig. 7).—The administration of meralluride, 160 mg. mercury equivalent (4 ml.), as a prime and 160 mg. as a sustainer produced a sodium excretion of 1,360 ± 170 μEq per minute which was maximum and stable at the end of 2 hours. At this point, 2,000 mg. acetazoleamide, both as a priming and a sustaining dose, produced an additional increase in sodium excretion to 1,742 ± 182 μEq per minute which was maximum and stable at the end of the second 2-hour period. At this point, a 500 mg. priming dose of chlorothiazide produced a third increase in sodium excretion to 2,249 ± 240 μEq per minute which was maximum and stable at the end of the third 2-hour period.

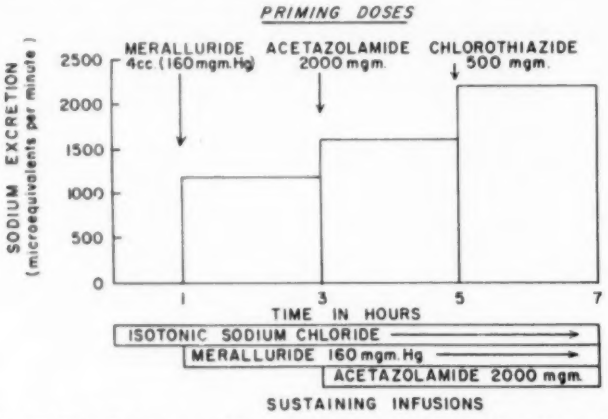

Fig. 7.—The effect of a combination of meralluride, acetazoleamide, and chlorothiazide on sodium excretion illustrating noncompetitive mechanisms. In spite of maximum sodium responses from meralluride and acetazoleamide, a small dose of chlorothiazide produces a third increase in sodium excretion.

The excretion of potassium was similar to that observed in previous studies and, in general, paralleled bicarbonate excretion. The excretion of bicarbonate was similar to that in previous studies and generally increased after acetazoleamide (35 per cent of the sodium rate) and declined after chlorothiazide was added to only 25 per cent of the sodium rate. Chloride excretion was also similar to that in previous studies and, in general, increased after meralluride to a degree similar to that of sodium. After acetazoleamide was added, chloride excretion rate decreased to about 50 per cent of the sodium rate. However, after chlorothiazide was added the chloride excretion rate once more increased to about 70 per cent of the sodium rate.

G. Renal Hemodynamic Effects.—Contemporary observations of glomerular filtration rate (inulin clearance) and renal plasma flow (para-aminohippurate clearance) revealed no significant changes (data not presented).

DISCUSSION

The synthesis and exploration of varying chemical compounds with diuretic action has encouraged speculation concerning their mechanisms of action. Admittedly, this area of man's knowledge is woefully inadequate due to insufficient techniques for study. Histochemical studies have furnished certain bits of information implicating specific enzyme system inhibition by mercurial diuretic therapy. Also, the ability of sulfonamides and other agents to inhibit the carbonic anhydrase system is fairly well documented by in vitro studies. These drug effects inhibit sources of energy for the activation of the ion exchange mechanism for sodium reabsorption. Although this information is valuable, it is incomplete and, furthermore, does not necessarily describe the specific electrolyte excretion effects to be expected from specific enzymatic inhibition.

The purpose of this study therefore, is to present indirect evidence concerning the mechanisms of action of 3 groups of diuretics. If one proceeds with the hypothesis that the primary action of diuretics is to inhibit the renal tubular reabsorption of sodium, changes in urinary sodium may afford indirect evidence of diuretic action. Furthermore, the relationships of the urinary excretion of various other electrolytes, especially potassium, chloride, and bicarbonate, under conditions of diuretic therapy may afford additional mechanistic information. The concept to be tested involves the "saturation" of the hypothetical receptor site, or the inhibition of one renal transport mechanism by the first drug to be followed by the second drug administration, and the observation of subsequent changes in sodium excretion. Dosages of the "second" drugs were selected to avoid the factor of potency (see Materials and Methods). If the second (or third) drug effected an additional increase in sodium excretion, one might speculate that a second (or third) receptor site or transport mechanism had been affected. If the second (or third) drug failed to effect an additional increase in sodium excretion, one might speculate that the receptor site or transport mechanism of the first drug had been "saturated" and that the second drug had no other site (or mechanism) through which to act and that their mechanisms were "competitive."

The results of this study suggest that the mechanisms of action (? enzyme system inhibition) of meralluride and acetazoleamide are different or noncompetitive. This conclusion is based on the significant increase in sodium excretion following the administration of acetazoleamide *after* a maximum excretory rate of sodium excretion has been produced by meralluride as a priming injection and during the continued administration of meralluride as a sustaining infusion. Additional evidence is furnished by the results of the study in which the order of drugs is reversed. A similar effect is observed when acetazoleamide is administered as a priming injection and sustained throughout the study. Although an equipotent (or less) dose of meralluride is then injected, there is still an increase in sodium excretion. In other words, massive doses of either acetazoleamide or meralluride cannot "block" the

effect of subsequent doses of the other drug (meralluride or acetazoleamide) on the excretion of sodium suggesting that a second receptor site has been acted upon, a second renal transport mechanism inhibited.

A slightly different conclusion must be drawn concerning the relationships of meralluride and chlorothiazide. Since chlorothiazide can further increase the excretion of sodium even after a maximal effect has been produced by meralluride, another mechanism or receptor site appears to be involved. The converse is not true, however. After a maximum effect has been produced by chlorothiazide, the addition of meralluride cannot further augment sodium excretion. This suggests that chlorothiazide and meralluride share a single mechanism of action or receptor site (for transport inhibition) but, also, that chlorothiazide can augment sodium excretion by inhibition of another transport mechanism. Meralluride apparently can augment sodium excretion by inhibition of only a single sodium reabsorptive transport mechanism. In other words, chlorothiazide can "block" the effect of subsequent doses of meralluride but meralluride cannot "block" the effect of subsequent doses of chlorothiazide on the excretion of sodium.

The relationships of acetazoleamide and chlorothiazide reflect a somewhat similar situation. Although chlorothiazide can further augment sodium excretion even after a maximum effect has been produced by acetazoleamide, the converse is not true. This suggests that these two drugs share one mechanism of action or receptor site but that chlorothiazide can exert its effects through yet another mechanism. In other words, chlorothiazide can "block" the effect of subsequent doses of acetazoleamide but acetazoleamide cannot "block" the effect of subsequent doses of chlorothiazide on the excretion of sodium.

That chlorothiazide does not necessarily use the "acetazoleamide mechanism" when blocked by meralluride and the "meralluride mechanism" when blocked by acetazoleamide, is indicated by the results of the final segment of this study. Although both transport mechanisms have been inhibited by the prior priming doses and continued sustaining doses of both meralluride and acetazoleamide, the subsequent injection of even a small dose of chlorothiazide evokes a further increase in sodium excretion. This suggests that chlorothiazide activity cannot be "blocked" by meralluride or acetazoleamide (singly or in combination) although it is capable of "blocking" both of them. This may be considered indirect evidence that chlorothiazide produces its effect in inhibiting the renal tubular reabsorption of sodium by a third mechanism of action which is noncompetitive with the mechanisms of action of meralluride and acetazoleamide. That this may indicate a greater range of activity of enzyme binding throughout the nephron by chlorothiazide must be considered, but the data at hand do not permit us to differentiate this point. It must be emphasized that these changes were observed without contemporary change in renal hemodynamics (glomerular filtration rate and renal plasma flow).

The relationships of other urinary electrolytes, such as potassium, chloride, and bicarbonate, lend further support to these conclusions. Detailed description of these changes have been omitted from the discussion since the primary consideration of the moment is that of diuretic action which we consider to be primarily natriuretic.

SUMMARY

Indirect evidence concerning the mechanisms of action of meralluride, acetazoleamide, and chlorothiazide as diuretics has been presented in data consisting of the urinary excretion of sodium, potassium, chloride, and bicarbonate. The primary action of these diuretics is the augmentation of the renal excretion of sodium.

The data suggest that the mechanisms of action of meralluride and acetazoleamide are noncompetitive and different, i.e., different "receptor sites" are acted upon or different renal transport mechanisms for the reabsorption of sodium are inhibited.

The mechanism of action of meralluride is competitive with that of chlorothiazide (the same). Yet, chlorothiazide apparently can act through another mechanism which is noncompetitive with meralluride but another mechanism is apparently not available to meralluride.

The mechanism of action of acetazoleamide is competitive with that of chlorothiazide (the same). Yet, chlorothiazide apparently can act through another mechanism which is noncompetitive with acetazoleamide.

The third mechanism of action as a diuretic exemplified by chlorothiazide cannot be inhibited by the concurrent administration of meralluride and acetazoleamide, i.e., the receptor site (for the inhibition of renal reabsorption of sodium) of chlorothiazide cannot be "saturated" by meralluride or acetazoleamide.

This additional mechanism of action of chlorothiazide may imply involvement of another enzyme system or it may imply a greater range of activity of chlorothiazide throughout the nephron.

We express our sincere gratitude to Drs. Robert Pitts, Karl Beyer, and Carroll Handley, for their review of this manuscript and for helpful comments.

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ON THE ZONE ELECTROPHORESIS OF HUMAN PAROTID SALIVA IN STARCH GELS

KIRK C. HOERMAN, D.D.S., M.S.*

WITH THE TECHNICAL ASSISTANCE OF V. J. BERZINSKAS**

UP TO 12 protein components have been demonstrated in pure human parotid saliva using boundary electrophoresis.[1-3] The role of salt anomalies, however, appears to be incompletely determined. Zipkin and associates,[2] in their studies of human parotid protein, drew attention to the fact that the major boundary, occurring at an average mobility of $+ 0.3 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1}, was not examined for contribution of the salt anomaly, which is known to exist at low mobilities in descending boundaries.

The principal advantage of zone electrophoresis would be the exclusion of boundary anomalies. Added to this would be better resolution of low molecular weight proteins, ability to handle smaller quantities of protein, and ease of isolation of distinct protein components. Nonetheless, no report has appeared claiming a zone electrophoretic separation of parotid protein comparable to that provided by the classic Tiselius methods.

Kinersly[4] was able to resolve whole human saliva protein into 2 or 3 zones on paper supports. In the light of the demonstration of some 9 to 12 distinct protein components in human parotid saliva—indeed a component part itself of total admixture whole saliva—by boundary electrophoresis, it would appear that zone electrophoresis is of little value in the study of salivary proteins. Our attempts to separate parotid saliva protein on paper supports were discouraging. Rarely could more than two zones of low mobility be resolved. This enigma led to an assumption that the major portion of human parotid protein was cationic and strongly adsorbed on paper. After examination of other solid media, starch gel supports were found suitable, provided rigid control of procedures was instituted.

METHODS

Generally, the technique of Smithies[5] for the separation of blood serum protein in starch gels was followed.

Parotid Saliva Collection.—The Lashley parotid cup, as modified by Curby,[6] was used to obtain pure parotid gland secretions. An accurately measured volume of not less than

From the Dental Research Department, Naval Medical Research Unit No. 3, Cairo, Egypt (Cairo Office of the Naval Attache, Department of State, Washington 25, D. C.).

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*Lieutenant Commander, Dental Corps, U. S. Navy.

**Dental Technician Chief, U. S. Navy.

5 ml. of parotid saliva, stimulated by chewing,* was collected from each of 10 healthy men, aged 25 to 36 years. One milliliter of native secretion was taken for total protein determination by the biuret reaction.

Preparation of Sample for Electrophoresis.—To the parotid sample was added 4 volumes of cold acetone (−17° C.) to precipitate protein. This mixture was refrigerated for 24 hours at temperatures below 0° C., after which centrifugation at 1,300 g (−7° C.) was accomplished. The supernate was decanted following a negative biuret color, and the precipitate was lyophylized. When ready for use, protein samples were reconstituted with an appropriate volume of distilled water, usually 0.2 to 0.5 ml.

Electrophoretic Conditions.—A 15 per cent solution of Merck reagent grade soluble potato starch (in boric acid, 0.03 M total borate, pH 8.4) was heated until complete eruption of starch grains occurred. Entrapped air was removed under negative pressure, and the solution was poured into Plexiglas trays of the dimensions suggested by Smithies.[5] The filled trays, covered with lubricated Plexiglas strips, were allowed to stand until a firm gel formed. A paper strip† was cut to fit the short axis of the gel slab and, soaked in reconstituted protein sample, was placed in a slit cut in the gel midway on the long axis. After insertion of the parotid protein sample, the area was covered wth a lubricated cover glass slip. The gel slab was set on the migration chamber and the ends brought in contact, by means of buffer-soaked paper wicks, with electrode basins filled with 0.3 M boric acid, pH 8.4 adjusted with NaOH. A Pt/Pt electrode system was used. The entire unit was placed in a cold room (4° C.) and a potential gradient of 8.7 volts per centimeter (4 to 5 Ma.) was applied. Five hours under these conditions gave satisfactory separations. The gel slabs were carefully removed from the trays: cut in half, longitudinally; stained in a concentrated solution of Amido-Black 10B dye; and washed in a series of methanol, acetic acid, and water baths according to the procedures of Smithies.[5]

RESULTS

Nine to twelve distinct protein zones were readily visible after staining the cut gel slabs. Fig. 1 shows a photograph of a typical stained gel slab with an accompanying drawn diagram of the various parotid protein zones. Six to nine components migrated as cations under the present electrophoretic conditions. Greatest variation in patterns occurred in the area of components VI and VIII. Zone VI usually revealed some spreading; however, subzones were frequently observed within the total area of VI. Components VII and VIII showed low dye-binding capacity. There was noticeable fading of these zones following one hour in washing solutions (methanol, distilled water, and glacial acetic acid: 50:50:10, v/v). This fading effect could be diminished by altering the washing solution to a ratio of 30:70:10, v/v. The anions (components I, II, and III) were present without variation.

Standardized photographs of stained gels were scanned in a recording densitometer (Aminco JLC) to obtain an approximate concentration curve of each zone. The resulting curve of one such scanning, along with a photograph of the stained gel, is shown in Fig. 2. The greatest concentration of cationic protein was found in the area from zones V to VII and constituted approximately 40 per cent of the total. The slowest migrating anionic protein (component III) contributed approximately 20 per cent of total protein.

It was observed that the anions, I, II, and III, along with cations V and VI, acquired a migration along the gel in the fashion of blood serum proteins.

*Kindly supplied by the American Chicle Company as Dentyne gum.

†The tightly pressed paper fillers in boxes of Johnson & Johnson dental root canal points were ideal for sample application.

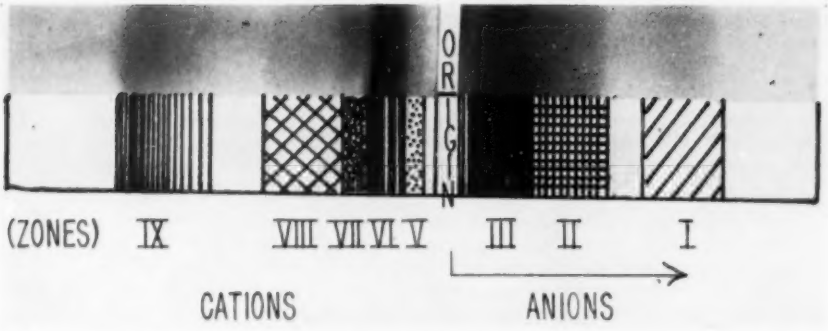

Fig. 1.—Photograph and drawn diagram of a stained gel slab with the typical parotid saliva protein component distribution shown. Total protein, 230 mg. per cent.

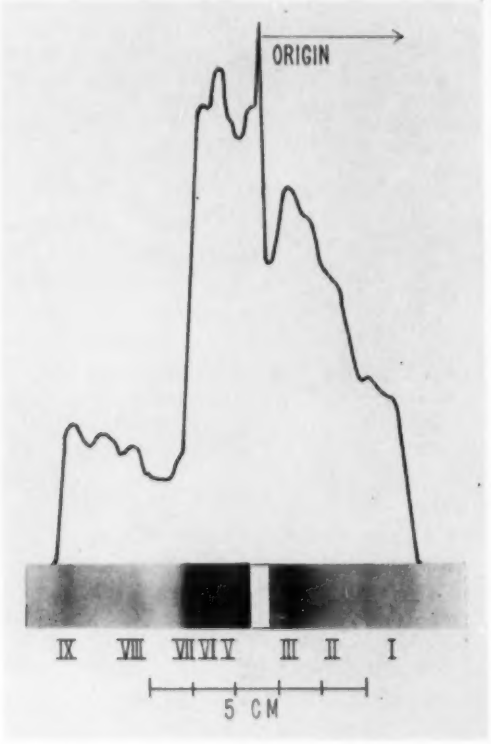

Fig. 2.—Photograph of a stained gel slab showing parotid protein distribution with an accompanying concentration curve approximated by means of a scan of a standardized photograph of the gel slab in an Aminco JLC photodensitometer.

Fig. 3 shows the result of an experiment in which a parotid protein and blood serum sample were inserted in the same gel slab and subjected to electrophoresis. Parotid components I and III exhibited migrations similar to serum albumin and beta globulin, respectively. However, information gained in such an experiment would serve merely as a guide to isolation and preparative procedures leading to absolute comparative identification of parotid components I and III.

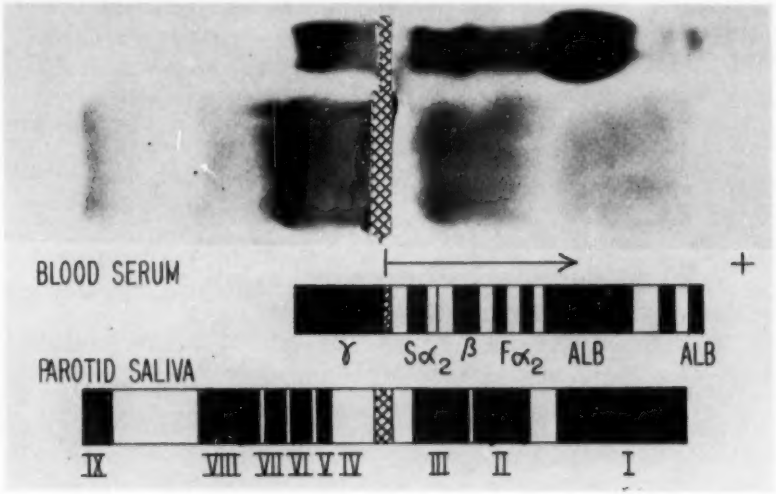

Fig. 3.—Photograph and explanatory diagram of a gel slab in which parotid saliva protein and blood serum were subjected to electrophoresis concomitantly.

DISCUSSION

Control of parotid amylase action on the starch gel support was accomplished in an acceptable fashion by employing a relatively chloride-free protein sample as a result of the acetone precipitation method, and electrophoresis runs at low temperatures with increased potential gradients. Some amylase activity usually occurred immediately off the point of sample insertion toward the cathode. It was of such an order, however, as to have no deleterious effect on the supporting medium and, in turn, on the migration of the faster-moving cationic components.

Changes in the physical properties of the starch gels after staining and washing prevented the accurate measurement of migration constants. And the indirect method of obtaining concentration curves from standardized photographs of the stained gels was considered of little quantitative value.

Among the advantages of the method are: (1) simplicity and convenience, (2) reduced volumes of native parotid secretion (protein) required, (3) possibility of characterization of distinct components by specific color reactions or application of radioactive isotopes, and (4) ability to isolate various protein components for further biologic classification.

SUMMARY

A method for separation of parotid saliva proteins by zone electrophoresis in starch gels has been presented. It was found simple, convenient, and free from the great technical requirements of classic boundary electrophoresis.

A study of 10 samples of parotid saliva subjected to electrophoresis by the present method revealed reproducible protein migration patterns. Nine to twelve protein components were regularly resolved. This finding was in good agreement with those reported in which boundary electrophoresis was employed. The majority of components migrated as cations at pH 8.4.

Two parotid protein components were observed to migrate in the fashion of blood serum proteins subjected to similar electrophoretic conditions. Absolute identification of these parotid components on a comparative basis awaits further investigation.

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RELATIONSHIP OF EQUILIBRIUM OF DISTRIBUTION, BIOLOGIC DECAY RATES, AND SPACE AND MASS OF H^3, Cl^{36}, AND Rb^{86} OBSERVED SIMULTANEOUSLY IN A COMFORTABLE AND IN A HOT AND HUMID ENVIRONMENT IN CONTROL SUBJECTS AND IN PATIENTS WITH CONGESTIVE HEART FAILURE

C. T. Ray, M.D.,* and G. E. Burch, M.D.**
New Orleans, La.

IN ORDER to study the demands placed on the vascular system by thermal regulation in a hot and humid environment, daily clinical studies were made of unselected patients admitted to adjoining wards at the Charity Hospital; one was maintained at comfortable conditions with air conditioning, and the other was exposed to the hot and humid environment of New Orleans in mid-summer. In most instances, even though the patients' impression and ordinary clinical observations readily convinced the clinician of the marked benefits of the comfortable environment, such impressions were not substantiated by differences in the pulse rates, character and rate of respiration, blood pressure, body temperature, and other physiologic phenomena between the two groups. Since a hot and humid environment profoundly influences sweating and water and electrolyte intake, as well as other phenomena concerned with thermal regulation, the details of certain aspects of electrolyte and water metabolism were investigated by tracer methods already in use in this laboratory to learn the influence of these two types of environments on sick people. Control subjects without disturbance in water and electrolyte metabolism were observed along with patients with chronic congestive heart failure.

Tritium, Rb^{86}, and Cl^{36} were employed simultaneously as tracers along with determinations of stable electrolytes under controlled metabolic conditions. In addition, the experiments were also designed to elucidate water and electrolyte metabolism in man by simultaneous observations of the 3 tracers and nontracer elements.

METHODS AND MATERIALS

Ten Negro women (see Table I for clinical data) were studied under controlled metabolic conditions in the wards of Charity Hospital during the hot and humid period of July 9 to Aug. 3, 1956. Five subjects were injected simultaneously with Cl^{36} (as

From the Department of Medicine, Tulane University School of Medicine, and the Charity Hospital of Louisiana, New Orleans, La.

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*Associate Professor of Medicine, Tulane University School of Medicine; Senior Visiting Physician, Tulane Unit, Charity Hospital of Louisiana, New Orleans, La.

**Henderson Professor and Chairman, Tulane University School of Medicine; Physician-in-Chief, Tulane Unit, Charity Hospital of Louisiana, New Orleans, La.

NaCl), Rb^{86} (as $Rb^{86}Cl$), and H^3 (as H^3OH) in doses shown in Table I. Metabolic data included intake and output of K^{39}, Cl^{35}, Na^{23}, and water. Previous studies had shown the loss of chloride, sodium, and potassium in normal stools to be negligible,[1, 2] and there were no instances of diarrhea among the subjects in this study. Body weight was recorded each morning for all subjects when the clinical state permitted.

TABLE I. CLINICAL DATA ON THE FIVE SUBJECTS STUDIED

PATIENT NUMBER	AGE (YR.)	DIAGNOSIS	DOSE OF ISOTOPES Cl^{36} (MC.)	Rb^{86} (MC.)	H^3 (MC.)	TREATMENT
1	46	Control, convalescent, upper respiratory infection	0.026	0.631	3.749	None
2	50	Control, tuberculous arthritis	0.027	0.663	4.104	Streptomycin, sodium para-amino-salicylate, body and leg cast
3	52	R.H.D., C.H.F.	0.020	0.481	2.993	Digitalis mercurial diuretics
4	52	A.S.H.D., H.H.D., C.H.F.	0.027	0.662	3.780	Digitalis mercurial diuretics
5	65	A.S.H.D., C.H.F., empyema, pneumothorax	0.026	0.642	3.852	Digitalis penicillin thoracentesis

Key to abbreviations: A.S.H.D. = arteriosclerotic heart disease; H.H.D. = hypertensive heart disease; R.H.D. = rheumatic heart disease; C.H.F. = congestive heart failure.

Samples of blood were collected daily at 8:00 A.M. prior to eating; one aliquot was collected under oil for studies of the plasma and another aliquot was used for studies of whole blood. Studies on the plasma included measurements of Cl^{36}, Rb^{86}, H^3, Cl^{35}, Na^{23}, K^{39}, creatinine, and osmolarity. Studies on the whole blood included measurements of Cl^{36}, Rb^{86}, H^3, Na^{23}, K^{39}, and hematocrit. The concentrations of Cl^{36}, Rb^{86}, and H^3 in the erythrocytes were computed by the following equation:

$$C_E = \frac{C_{WB} - (1 - H)\ C_P}{H}$$

where C_E = the concentration of the substance in erythrocytes; C_{WB} = the concentration of the substance in whole blood; C_P = the concentration of the substance in plasma; and H = the hematocrit.

The urine was collected under toluene from 8:00 A.M. to 8:00 A.M. of the following day. The volume of urine was recorded along with specific gravity, pH, osmolarity, and Na^{23}, Cl^{35}, Cl^{36}, Rb^{86}, K^{39}, H^3, and creatinine.

All samples of whole blood were laked by freezing and thawing before radiometric or chemical analyses were made. Sodium and potassium content of whole blood, serum, and urine was determined by flame photometry, chloride by the method of Schales and Schales,[3] osmolarity of serum and urine by the Fiske osmometer, and creatinine by the method of Haugen.[4]

The concentrations of Rb^{86} and Cl^{36} were determined by the method previously described.[5] Separation of the two isotopes in the same sample was achieved by counting a few hours after collection of the samples and then recounting 200 days later after the Rb^{86} had decayed. Radioassay for tritium was preceded by distilling the water from

whole blood, urine, and serum at 90° to 100° C. The distillate contained no Cl^{36}. Radioassay of the tritium in this water distillate was done with a Packard Tri-Carb liquid scintillation counter.*

The subjects were divided into equal groups: one group was observed in the comfortable air-conditioned ward (mean temperature 74.7° F., range 70.1° to 80.2° F., and a mean relative humidity of 73.4 per cent with extremes of 90 per cent and 60 per cent), and the other group was in a sub-tropical summer environment of an ordinary ward in July. The average daily temperature recorded twice daily on the ward was 86.8° F. with a relative humidity of 62.0 per cent. For the period of observation the Weather Bureau reports revealed a mean temperature of 83.4° F., range 94° F. to 69° F., and a mean relative humidity of 80.0 per cent, range 98 per cent to 47 per cent. The maximum daily temperature was 90.0° F. or greater on 21 of the 26 days of observation. At the end of approximately 2 weeks the subjects were changed to the other environment.

RESULTS

For convenience of presentation the results summarized in Figs. 1 to 6 and Tables II and III are divided into 3 sections: equilibrium of distribution, biologic decay rates, and measurements of space and mass.

Equilibrium of Distribution.—Previous experiments[6-8] indicated that it was not possible to determine in intact man the moment at which equilibrium of distribution of radioelements is established. Since only a limited number of isotopic compartments of the body could be sampled simultaneously, equilibrium of distribution of Cl^{36}, Rb^{86}, and H^3 was estimated from the following: (a) the constancy of slope of the concentration-time course curves of the tracers, (b) the constancy of the magnitudes of the calculated space and mass of the nontracer elements, and (c) the specific activity of more than one compartment, namely, plasma and erythrocytes for Rb^{86}. The values noted below were derived from these 3 methods.

Radiochloride: Equilibrium of distribution of Cl^{36} probably did not occur until at least 3 to 4 days following injection of the tracer (Figs. 1, 2, 3, 5, and 6). There were no detectable differences between comparable subjects at different environmental temperatures; however, the presence of congestive heart failure tended to delay the development of equilibrium of distribution of Cl^{36} in both environments.

Radiorubidium: It is probable that equilibrium of distribution of Rb^{86} was not established at any time during these observations and certainly not for the initial 7 to 11 days. This was supported by the fact that the concentration of Rb^{86} in the erythrocytes was increasing while that in the plasma was declining for the first 7 to 11 days after the injection of the tracer. No effect of environment or congestive heart failure could be detected in these concentration-time course curves of Rb^{86}.

Tritium: The concentration-time course of tritium in plasma and erythrocytes showed that equilibrium of distribution was established within 24

*The authors wish to express their appreciation to Drs. Leon Jacobsen and George Okita and associates, University of Chicago School of Medicine, for the use of their laboratory facilities for counting the tritium samples.

hours. Since equilibrium occurred in all cases before the first samples of blood were collected, no effect of environment or congestive heart failure could be noted.

Comment: The subjects were allowed to drink water as desired and eat a regular diet of known electrolyte content. The daily intake of the nontracer material was sufficient to exceed the increased requirements produced by the stress of the hot and humid environment so that the influence of the environment was not made evident in the rate of development of equilibrium of distribution of Cl^{36}, Rb^{86}, and H^3. Had the intake of nontracer materials been inadequate to meet these increased demands with resultant functional sequestration of electrolyte compartments in the body, then impairment of the development of a state of equilibrium of distribution might have been expected. Congestive heart failure, on the other hand, with impairment of the steady state of metabolism for Cl^{36}, Rb^{86}, and H^3 might be expected to interfere with equilibrium of distribution. This was evident for Cl^{36} only.

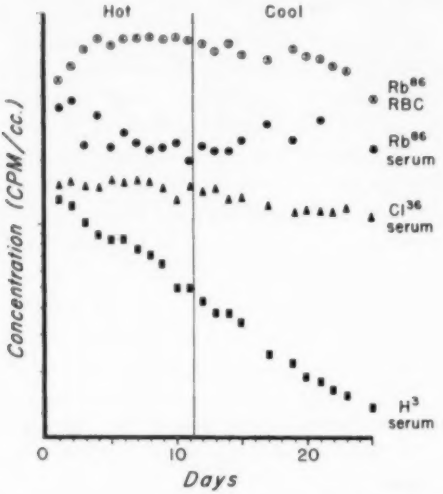

Fig. 1.—Biologic decay rates of Rb^{86}, Cl^{36}, and H^3 in Subject 5 with congestive heart failure. The concentration-time course curves are shown for Rb^{86} in the erythrocytes and serum, Cl^{36} in the serum, and H^3 in serum in that order from above downward. The ordinate is logarithmic in this and subsequent figures. The numerical value of concentration of these radioisotopes is not shown since only the rate of decline in concentration is considered. The vertical line indicates the day of study on which the environment was changed.

Since Rb^{86} entered the potassium metabolic pool in the body, the isotope became predominantly intracellular. Therefore, unless the hot and humid environment or congestive heart failure caused sufficient alterations in the rate of the metabolic processes of K^{39} in cells, the environmental conditions would not be expected to modify the rate with which equilibrium of distribution of Rb^{86} was approached. The slow rate of exchange of some of the cellular compartments is reflected in the time required for the concentration of the Rb^{86} to reach a maximum level in the erythrocytes. The importance of the slow rate

of uptake as well as the lag in release of intracellular electrolyte back into the plasma in estimating the time of equilibrium of distribution has been previously indicated.[8-11] As long as the time course curves of "specific activity" in different compartments are not parallel, equilibrium of distribution cannot exist. Furthermore, lags in exchange of the tracer result in inconstant calculated values for space and mass with time. The dependence of the concentration of Rb^{86} in the plasma on the "feed-back" from cellular compartments in general is indicated by the parallel decline in concentration of Rb^{86} in the plasma and erythrocytes in the latter part of these experiments (Figs. 1 to 3).

Tritium was studied in two compartments, plasma and erythrocytes. There was no difference in concentration of H^3 in these two compartments by the time the first sample of blood was withdrawn, i.e., 20 to 22 hours after injection. The concentration-time course of H^3 in plasma revealed a constant rate of decline from the first measurement throughout the duration of these experiments. Since there were large unmeasured extraurinary losses of water via skin, respiratory tract, and stool, the values of H^3 space show a continuing expansion. Therefore, a time course curve of space and mass of H_2O was not satisfactory for estimating the time at which equilibrium of distribution developed.

Numerous studies of the time required for equilibrium of distribution of deuterium and tritium to develop in animals have been reported.[12-19] There is considerable evidence that equilibrium of distribution of H^3 develops rapidly in man, although very little published data are based upon experiments extending as long as 24 hours after injection of the tracer.[20-29] The rapid rate at which equilibrium of distribution of the isotopes of hydrogen develops indicates its distribution is primarily with the hydrogen of the water of the body. The exchange is slow with the other hydrogen atoms of the body.[13-17, 30-33] In chemical reactions involving hydrogen such as in enzymatic systems, there is preference for ordinary hydrogen over its two heavier isotopes.[13-17, 30-32, 34, 35] The amount of heavy isotope involved in such chemical linkages is a very small portion of a single injection, probably not amounting to over 1.5 to 2 per cent of the total dose. Since such a small amount is involved in slow exchanges, in both incorporation and in release from relatively stable chemical bonds, the effects of the isotopes are not evident in tracer studies of this type.

Biologic Decay Rates.—The biologic decay rates were determined as previously described.[6, 8] $C_{½}$ values were obtained for 5 subjects and $U_{½}$ was determined for 4 subjects. These parameters have the following connotations:

$C_{½}$ represents the length of time required for the concentration of the tracer to decline to one-half concentration in the compartment concerned at any time following equilibrium of distribution. $C_{½}$ values in these experiments were determined for the plasma compartment for Cl^{36}, Rb^{86}, and H^3 and in the erythrocyte compartment for Rb^{86}. $U_{½}$ represents the length of time required to recover one-half the administered isotope in the urine.

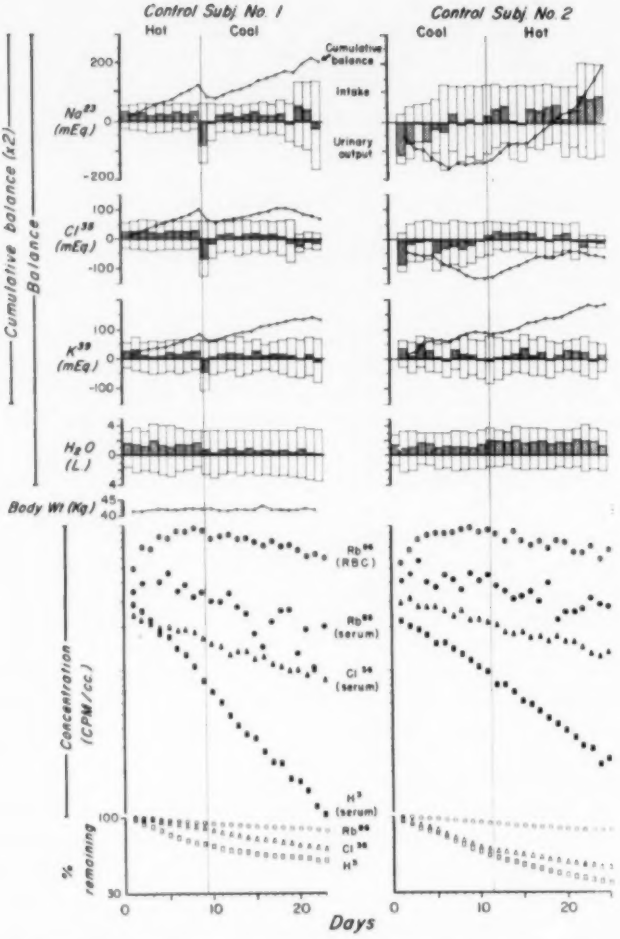

Fig. 2.—Biologic decay rates of Rb^{86}, Cl^{36}, and H^3 and metabolic balance data on 2 control subjects. The concentration-time course curves are presented as in Fig. 1. In the lower portion of the graph the per cent remaining in the body (not recovered in the urine) is expressed as a percentage of the injected dose of radioisotopes. In the portion of the graph showing electrolyte and water balance intake is indicated by columns above the base line, urinary output by columns below the base line, and balance by the solid columns. When balance was positive, the solid column is above the base line. The ordinate value for the cumulative balance is twice the values for the other balance data.

The average values obtained for the 3 parameters are summarized in Table II and in Figs. 1, 2, and 3.

TABLE II. THE INFLUENCE OF ENVIRONMENT ON BIOLOGIC DECAY RATES OF Rb^{86}, Cl^{36}, AND H^3

BIOLOGIC DECAY RATES (DAYS) C ½

SUBJECT	Cl^{36}			Rb^{86} SERUM			Rb^{86} ERYTHROCYTES		H^3		
	ENTIRE STUDY	HOT	COLD	ENTIRE STUDY	HOT	COLD	DESCENDING CURVE ONLY*	ENTIRE STUDY	ENTIRE STUDY	HOT	COLD
1	14.9	15.5	14.6	28.0	338.1	18.4	36.8	86.0	4.6	4.5	4.6
2	18.8	20.2	17.1	27.2	18.3	84.9	32.0	66.3	7.5	7.0	8.4
3	22.8	17.9	25.9	57.2		9.6	42.9	82.0	7.0	8.1	6.6
4	28.0	27.5	28.8	37.8	175.9	18.0	22.1	53.7	6.6	5.2	9.1
5	46.8	382.9	28.8	16.9	6.1		16.8	36.1	7.3	7.1	7.5
Mean	26.2	92.8	23.0	33.4	134.6	54.3	30.1	64.8	6.6	6.4	7.2

U ½

SUBJECT	Cl^{36}			Rb^{86}			H^3		
	ENTIRE STUDY	HOT	COLD	ENTIRE STUDY	HOT	COLD	ENTIRE STUDY	HOT	COLD
1	31.5	34.3	29.9	74.9	66.8	81.2	23.1	14.2	38.6
2	21.3	32.1	14.9	68.7	78.0	59.5	16.5	22.6	12.3
3	96.7†	99.4†	88.6†	58.2†	60.7†	57.4†	20.6†	29.9†	18.7†
	17.6			53.1			17.4		
4	46.0†	54.8†	38.9†		122.9†	87.1†		61.2†	23.7†
	39.2	39.5		101.9	117.5		34.2	49.8	
5	‡								
Mean	48.9	55.2	43.1	75.9	82.1	71.3	23.6	32.0	23.3

SUBJECT	PER CENT RECOVERED IN URINE		
	Cl^{36}	Rb^{86}	H^3
1	39.8	19.4	50.0
2	55.8	21.0	65.1
3	59.1	24.0	56.7
4	36.0	15.8	40.0
Mean	47.7	20.0	53.0

*Calculated from that part of the concentration-time course curve which followed the peak.

†This signifies figures calculated without mercury.

‡Incomplete urine collections.

Radiochloride: The $C_{½}$ and $U_{½}$ values for chloride were less for the control subjects than for the subjects with congestive heart failure. There was no definite effect of the two environments on these parameters for Cl^{36} in any of the subjects studied. Most of the chloride excreted by Subject 3 was in response to mercurial diuretics so that effects of the environment could have been masked (Fig. 3 and Table II).

Comment: The influence of the daily intake of Cl^{35} on biologic decay rates of Cl^{36} has been demonstrated previously.[6, 36] When the subject is in a steady state, the $C_{½}$ may be estimated from the mass of stable element in the body and the dietary intake. In these experiments, the daily intake varied from 2.13 to 3.59 per cent of the average amount of stable element calculated to be in the body (Table III). The predicted $C_{½}$ values for Cl^{36} in the plasma for Subjects 1, 2, 3, and 4 were 20.0, 23.2, 19.3, and 32.6 days, respectively,

while the observed values were 14.9, 18.8, 22.8, and 28.0 days, respectively. These subjects, especially those with congestive heart failure, were not in a steady state since body weight changed.

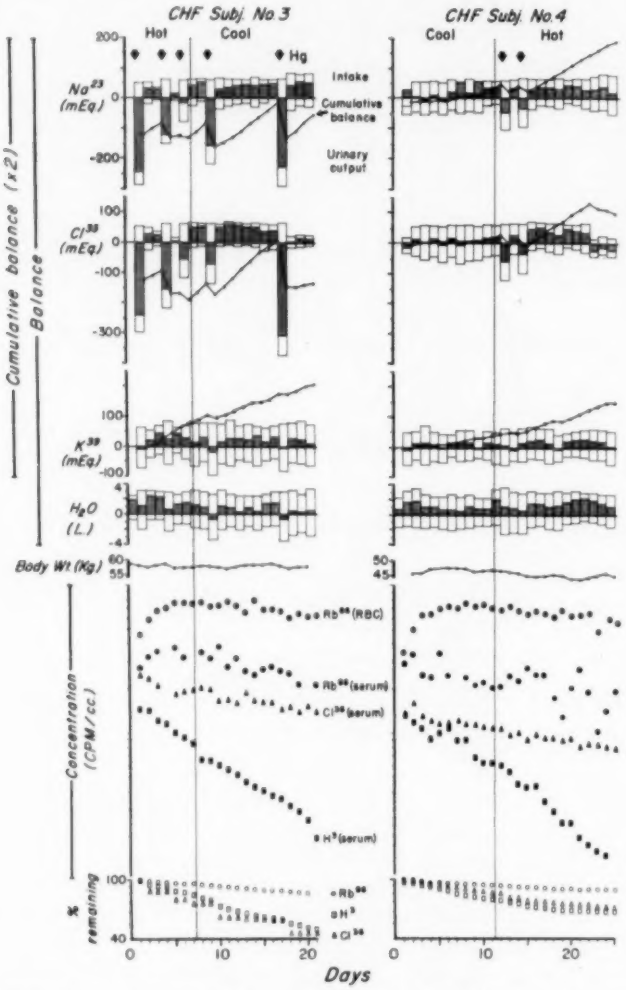

Fig. 3.—Biologic decay rates and metabolic balance data on 2 subjects with congestive heart failure. Manner of presentation is the same as in Fig. 2.

The $C_{\frac{1}{2}}$ values of Cl^{36} for the control subjects differed slightly from those with congestive heart failure. Since the daily intake of chloride was more than adequate to meet the demands of the hot environment, there were no significant differences in $C_{\frac{1}{2}}$ or $U_{\frac{1}{2}}$ values in the two environments. The effect of

mercurial diuretics on $U_{½}$ is illustrated in Table II. Subject 3, for example, excreted most of the chloride during mercurial diuresis and thus had a $U_{½}$ value of 17.6 days when the entire study was considered and 96.7 days when the days of mercurial diuresis were excluded.

TABLE III. RELATIONSHIP OF INTAKE TO CALCULATED MASS OF WATER, CHLORIDE, AND POTASSIUM

	WATER							
	S_o, H^3 (0 TIME)		S_t, H^3 (22 HOURS)		CALCULATED BODY WATER*		INTAKE	
SUBJECT	L.	PER CENT B.W.	L.	PER CENT B.W.	L.	PER CENT B.W.	L.	PER CENT 22 HR. S_t
1	27.4	66.3	31.8	77.2	22.6	54.8	3.43	10.8
2	28.2	40.3	28.0	40.0	37.0	52.8	3.41	12.2
3	27.1	44.9	31.2	51.7	29.6	49.0	2.93	9.4
4	35.6	77.6	37.5	81.6	27.6	60.1	2.92	7.8
5	21.3		22.1					
Mean	27.9	57.2	30.1	62.6	29.2	54.1	3.2	10.1
	CHLORIDE				POTASSIUM			
	M_t, Cl^{36}		INTAKE		M_t, Rb^{86}		INTAKE	
SUBJECT	M_t (GM.)	PER CENT B.W.	GM.	PER CENT M_t	M_t (GM.)	PER CENT B.W.	GM.	PER CENT M_t
1	56.0† 72.6	0.152	1.94	2.67	144.0	0.344	2.48	1.72
2	63.6	0.093	1.90	2.99	117.0	0.172	2.63	2.25
3	53.0	0.091	1.90	3.59	104.4	0.179	2.92	2.80
4	88.8	0.193	1.89	2.13	143.9	0.313	2.17	1.51
5	51.5							
Mean	62.6	0.132	1.91	2.85	127.3	0.252	2.55	2.07

*Volume of body water calculated by the following formula: $M_o = \frac{I \times C_{½}}{0.693}$

†Average of first 4 days.

Radiorubidium: The $C_{½}$ and $U_{½}$ values for Rb^{86} show no effect of environment nor is there any obvious relation of these parameters of Rb^{86} to those of Cl^{36} (Table II). The concentration of Rb^{86} in the serum of all subjects varied considerably. The ratios of concentration of Rb^{86} in the erythrocytes to that in the serum are shown in Fig. 4.

The concentration-time course curve of Rb^{86} in the erythrocytes revealed an increase in concentration of Rb^{86} for the first 8 to 11 days of observation. The $C_{½}$ value for the erythrocyte compartment was therefore expressed in two ways (Table II): (1) the $C_{½}$ value for the descending portion of the concentration-time course after the peak concentration had been reached and (2) the $C_{½}$ value for erythrocyte compartment in which the ascending portion of the curve was included neglecting equilibrium of distribution. The value by the first method was essentially one-half as long as that by the second method.

Comment: By definition of $C_{½}$, a state of equilibrium of distribution must first exist before this parameter can be measured. This state of equilibration probably never exists completely for an isotope of potassium or Rb^{86}. The error introduced by assuming that equilibrium of distribution was established is probably minimal between the fifth and twelfth days after injection[2, 8, 11]; however, the magnitude of the error is unknown.

Since the $C_{½}$ value for Rb^{86} based upon the entire concentration-time course curve in the erythrocytes was approximately twice that based upon the descending portion alone, the rate of exchange of Rb^{86} between erythrocytes and plasma must have remained essentially constant throughout the entire observation. Furthermore, when considering exposure of a specific compartment to the radiations of a radioactive element, the $C_{½}$ value derived from the entire concentration-time course curve of that compartment must be utilized.

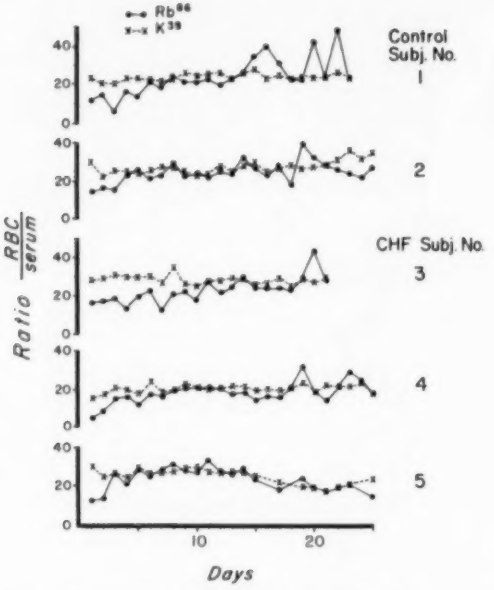

Fig. 4.—Time course of the ratios of K^{39} in the erythrocytes to that in the serum, and the ratios of Rb^{86} in the erythrocytes to that in the serum in each of the 5 patients. In only one subject (C.H.F. Subject 3) was there a prolonged lag in the time required for the ratio of Rb^{86} to approach the value of the ratio of K^{39}.

The concentration of Rb^{86} in plasma depends principally upon the rates with which various tissues take up and release Rb^{86}. Since the rate of decline in concentrations of Rb^{86} in the plasma was quite similar to that for the descending portion of the concentration-time course curve in the erythrocytes, it is apparent that the concentration in the plasma is dependent on release of Rb^{86} from organs. Furthermore, since only about 15 to 24 per cent of the injected Rb^{86} was recovered in the urine and the $C_{½}$ of plasma and erythrocyte compartments were similar and the ratios of Rb^{86} and K^{39} in the erythrocyte and that in the plasma were similar (Fig. 4), it is probable that the erythrocyte compartment reflects the kaliokinetic phenomena of the large cellular compartment of the body. The metabolic processes, which are concerned with exchange of Rb^{86} and potassium between the plasma and cells, were not influenced by the climatic conditions of this study.

The influence of intake of the stable element, K^{39}, on the biologic decay rate of Rb^{86} is shown in Tables II and III. Since the ingested K^{39} does not equilibrate quickly with the K^{39} and Rb^{86} of the entire body before excretion, the effect of varying intake of K^{39} on biologic decay rates would not be as predictable as for a substance which equilibrates more quickly such as chloride or water. Nevertheless, increase in intake of potassium reduced the $C_{½}$ values of Rb^{86}.[8] In the subjects whose average daily intake of potassium was a greater percentage of the calculated potassium mass in the body, a shorter time would have been required to recover one-half of the injected Rb^{86} in the urine (Tables II and III). The administration of mercurial diuretics had little effect on urinary excretion of Rb^{86}.

Tritium: The biologic decay rates of tritium are shown in Figs. 1 to 3 and in Table II. There were no significant differences in $C_{½}$ values among 4 of the 5 subjects studied; however, one control subject had a small $C_{½}$ value. There was no consistent effect of environmental temperature on these values. The decay in concentration of tritium was remarkably constant throughout the observations. The time course of the per cent of the tracer remaining showed a decrease in slope toward the end of the experiments (Figs. 2 and 3), a behavior expected because of unmeasured extraurinary losses of tritium.

Comment: The values of $U_{½}$ were larger than those for $C_{½}$ because of extraurinary losses of water and tritium. Therefore, the $C_{½}$ value was a better index of water turnover than $U_{½}$. All subjects were allowed water as desired. Although no purposeful water loading was attempted in these studies, Control Subject 1 had a very large daily intake of water and maintained a water diuresis throughout the study. The smallest $C_{½}$ value was obtained in this subject. The effect of intake of the nontracer water on biologic decay rates of H^3 would be expected as in the case of any isotope with which the ingested stable substance reaches a state of equilibrium of distribution in the body.[6, 37]

The progressive decrease in the slope of the time course of the per cent of H^3 remaining in the body (not recovered in urine) could be due to fixation of H^3 in stable positions in the tissues. The amount of tritium which would be expected to be bound in such stable chemical states would be very small during the course of the present studies, and would probably be within the small error of radioassay of H^3. Observations on animals[12-17, 30-33, 37, 38] have demonstrated the capacity of H^3 to be bound in stable positions and to lag thereafter in exchange with the hydrogen of plasma water. If such stable binding involved a significant percentage of the injected tritium, one would expect the latter part of the concentration-time course curve to show a slower rate of decline than the first part of the same curve since the percentage of H^3 remaining in the body which is chemically bound would increase progressively with time. In these studies, there were no apparent changes in the rate of decline of the concentration-time course curves in plasma (Figs. 1, 2, and 3). Although tritium traces hydrogen in the body with some "isotope effects," since the great mass of readily exchangeable hydrogen in the body is in the body water, the concentration-time course curves and biologic decay rates in these studies reflected turnover of body water.

The effect of environmental heat on the rate of loss of water from the body as sensible and insensible water loss is well known. Figs. 1 to 3 and Table II fail to show any detectable differences between the effects of the hot environment and the air-conditioned ward on the biologic decay rates of H^3. This does not mean that heat is unimportant in water loss from the body and in water turnover. Subjects 1 to 4 ingested approximately the same amounts of water each day in both hot and cool environments. When in the hot environment the decrease in volume of urine was offset by an increase in nonurinary loss of water so that water turnover, with approximately equal daily intake in both environments, would be expected to be the same. The $C_{½}$ values in these observations were between the extremes reported by Pinson[37] which reflects differences in water intake. As noted previously,[6, 39] the biologic half-life of an isotope which is freely exchanging in a compartment can be shortened by increasing the intake of the nontracer substance in that compartment.

Space and Mass.—Because of the long physical half-life of Rb^{86} and Cl^{36}, it was possible to calculate the time course of space and mass of chloride and potassium, assuming Rb^{86} traces K^{39} adequately. Calculations of the time course of tritium space were not justified because of the failure to measure nonurinary losses of H^3. The water space measured by tritium dilution was calculated for the time of injection and for 20 to 24 hours later. In Subject 5, incomplete collections made calculations of the time course of space and mass inaccurate.

The calculated electrolyte space and mass of the traced substance were defined as follows[39]:

S_o = calculated tracer space or volume expressed in sampled compartment volume equivalents at t_o. This may be expressed in serum volume equivalents, erythrocyte volume equivalents, etc.

S_t = calculated tracer space at any time t.

M_o = total calculated exchanging nontracer mass at t_o.

M_t = total calculated exchanging nontracer mass at any time t.

These values were calculated from the following equations:

$$S_o = \frac{\text{amount of isotope injected}}{\text{concentration of isotope in sampled compartment at } t_o}.$$

$$S_t = \frac{\text{amount of isotope remaining in body at time t}}{\text{concentration of isotope in sampled compartment at time t}}.$$

M_o = calculated space (S_o) × concentration of nontracer in sampled compartment at t_o.

M_t = calculated space (S_t) × concentration of nontracer in sampled compartment at time t.

The results are summarized in Table III and in Figs. 5 and 6. In Table III, the calculated space and, therefore, calculated mass of body water was also obtained from the following formula: $M_o = \frac{I \times C_{½}}{0.693}$ where I is the daily intake of water.

The values for chloride mass and intake shown in Table III are averages over 20 to 25 consecutive days. Since Subject 2 was in a heavy body and leg cast, an accurate daily body weight was not available. Her weight was estimated to remain at 150 pounds.

The calculated values of potassium mass shown in Table III are averages of a number of daily M_t values obtained after the concentration time course of Rb^{86} in erythrocytes had achieved its maximal value. The days of the study included in these averages were as follows: Subject 1, ninth to fifteenth day; Subject 2, ninth to fifteenth day; Subject 3, thirteenth to twenty-sixth day; and Subject 4, eleventh to twentieth day.

Tritium: The tritium space at t_o and at 22 hours calculated by dilution of tritium and the average mass of body water calculated from the average intake of water and $C_{½}$ of H^3 varied as much as 20 per cent in the same subject (Table III). No one method of calculation gave consistently higher or lower values. The calculated body water varied from 54.8 to 77.2 per cent of body weight for Subject 1, 40.0 to 52.8 for Subject 2, 44.9 to 51.7 for Subject 3, and 60.1 to 81.6 per cent body weight for Subject 4.

Comment: The relatively large variations in calculated body water based on tritium dilution have been encountered by others.[12, 28, 37] The same variability was found with antipyrine and deuterium.[24, 40-43] The validity of the dilution techniques has been checked by desiccation techniques and determination of specific gravity of the body.[44] The reported wide variations are unreasonable. There is less water in fat than in other tissues, but it is difficult to explain the variations of body water from 40 to 80 per cent body weight on the basis of fat content alone. The average values for the 3 methods of calculation on the 4 subjects in this series were 57.2, 62.6, and 54.1 per cent, respectively, of body weight. The mechanism for the large day-to-day variations in a single individual is likewise difficult to explain. Subject 2 was rather obese which may explain the relatively low value for body water but Subject 3 was quite thin and had edema and a considerable amount of ascitic fluid yet the calculated volume of body water was small. These variations indicate the difficulties associated with the tracer method of calculating space and mass of a substance in the body of man. There are very little data on reproducibility of body water measurements with tritium.[37] The extreme variability of calculated body water which has been reported may in part be related to the use of a single measurement of the tritium content of serum at the end of several hours after the injection of the tracer. By extrapolating concentration time course curve based on numerous observations obtained over 1 to 2 days back to time of injection, the variability associated with a single determination should be avoided. A longer period of study would be satisfactory only if extrarenal losses of water were measured.

Chloride: The time courses of space (S_t) and mass (M_t) of chloride are shown in Figs. 5 and 6. The average daily mass expressed as per cent body weight, intake of Cl^{35} in both grams and as a per cent of average

M_t is shown in Table III. The time courses of calculated chloride space and mass showed a few variations in the first 3 to 4 days but thereafter both remained fairly constant. In Control Subject 1, there was a progressive rise in both calculated space and mass. The mass increased from an initial value of 52.9 Gm. at 24 hours to 91.7 Gm. at the end of 22 days reflecting extrarenal losses of Cl^{36} and Cl^{35} as well as inaccuracies of such measurements.

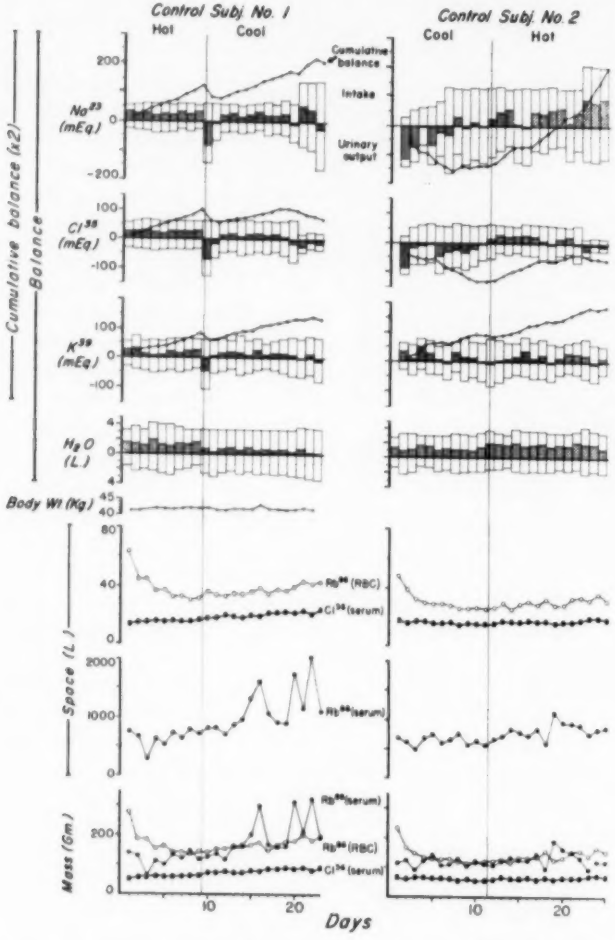

Fig. 5.—Time course of calculated space and mass of K^{39} and Cl^{36} in 2 control subjects. The data on electrolyte and water balance are presented as in previous figures. The time course of space of Rb^{86} (and therefore K^{39}) was calculated from the concentration of Rb^{86} in both the erythrocytes and serum. The calculated mass of K^{39} was also calculated from the Rb^{86} content of both compartments. The calculation of both space and mass of K^{39} is based on the assumption that Rb^{86} traces K^{39} adequately to justify such calculations. Both space and mass of Cl^{35} were calculated from the content of Cl^{36} in the serum.

There was no apparent effect of environmental temperature on the time course of S_t and M_t of chloride. The daily positive balance of Cl^{35} was greater in the hot environment which was most probably due to unmeasured extraurinary losses of Cl^{35} in sweat.

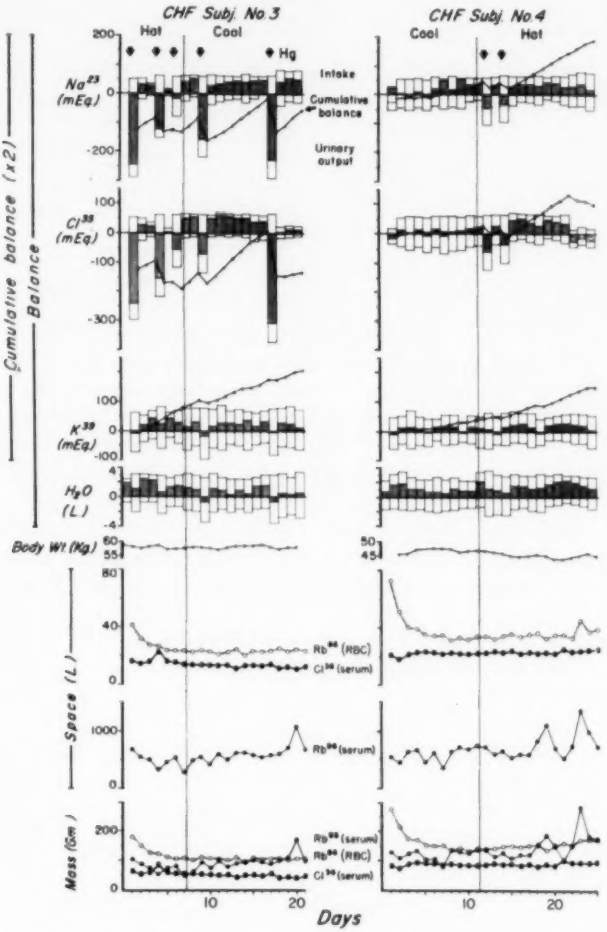

Fig. 6.—Time course of calculated space and mass of K^{39} and Cl^{35} in 2 subjects with congestive heart failure. The manner of presentation is the same as in Fig. 5.

Mercurial diuresis resulted in variations in calculated space and mass (Fig. 6, Subject 3). The effects of administration of $NaHCO_3$ during the last 3 days of the study are shown in Figs. 5 and 6.

Comment: In general, calculated space and mass of chloride bore a direct relationship to space and mass of body water in the control subjects and

those with heart failure (Table III). The average chloride space as a per cent of body weight was 46.1 for Subject 1 (37.8 for the first 5 days), 23.5 for Subject 2, 24.5 for Subject 3, and 50.4 for Subject 4.

Radiorubidium: The time course of calculated space (S_t) and mass (M_t) of potassium for both erythrocyte and plasma compartments are shown in Figs. 5 and 6.

The time course curves of calculated space and mass based on the erythrocyte compartment showed a decline for the first 7 to 10 days followed by a fairly constant value. The time course of S_t and M_t based on the plasma compartment shows rather wide daily fluctuations, although in general these curves were parallel to those based on the erythrocyte compartment. There was no apparent effect of the hot or cool environment on these parameters in any of these subjects.

In all subjects there was an expected progressive increase in the cumulative positive balance of K^{39} due to extrarenal losses. The calculated space and mass of K^{39} varied directly with that for water and chloride.

Comment: These studies add further to the data which show Rb^{86} to trace K^{39} fairly well in intact man.[2, 8, 45] There is no way to determine precisely when equilibrium of distribution of Rb^{86} has occurred. However, by studying the major compartments of K^{39}, such as the intracellular or erythrocyte compartments, a better estimate of the mass and kinetics of the traced element is obtained. The plasma and extracellular compartments are relatively small and, therefore, likely to vary considerably in concentration of Rb^{86}. The general parallelism between the time course of S_t and M_t based upon Rb^{86} in both erythrocyte and plasma compartments indicates the dependence of the plasma compartment on release of Rb^{86} from the major (intracellular) compartment. In only one patient (Subject 3) was there a long lag in the time required for the time course curve of M_t based on plasma compartment to reach the level of the curve based on erythrocyte compartment (Fig. 6).

The cellular metabolic phenomena which are related to the incorporation of Rb^{86} and potassium into the cellular compartment were not altered by environmental temperature during these studies. The rates of exchange of Rb^{86} between the cellular compartment and the plasma continued at the same rate throughout the experiment.

DISCUSSION

For the first time simultaneous studies of the kinetics of tritium, rubidium and chloride as well as aspects of the metabolism of sodium, chloride, potassium, and water have been made in man under comfortable and hot and humid environmental conditions to which both healthy and sick people of many areas of the world may be subjected. These studies failed to reveal any definite differences attributable to the environmental temperature in either the control subjects or in the patients with chronic congestive heart failure. In spite of this, however, except for one patient (Subject 4) they

were all impressed by the benefits of the comfortable environment upon their well-being and by the oppressiveness of the hot and humid environment. This was particularly true of the patient in the heavy body and leg cast. The attendants, physicians, and investigators were likewise impressed by these differences. More clinical data will be published elsewhere concerning these reactions. That over-all differences existed and the fact that the patients perspired less, were more relaxed, and clinically better was evident in spite of the experimental data. The psychologic reactions of the patients to these environments are especially in need of study.

However, in spite of these clinical differences, these detailed studies of metabolism of electrolytes and water failed to reveal any definite differences in equilibrium of distribution, biologic decay rates, or calculated space and mass of electrolytes and water. This is not surprising in view of the precipitous decline in the urinary excretion of sodium and chloride even though there is measurable change in the Na or Cl concentration in the plasma when a subject is placed on a low dietary intake of salt. In other words the regulation of any single physiologic phenomenon may be so sensitive that important differences in physiologic reactions can exist with immeasurable differences in the regulating phenomena responsible for the changes. To know that this is true for the metabolic and kinetic biologic phenomena of water and electrolytes is important.

The greatest differences, although not too large in magnitude, were noted in certain aspects of renal excretory function which will be described elsewhere.[47] It was rather interesting that the group of subjects under study drank large quantities of water each day. The intake far exceeded the needs for the comfortable environment and was more appropriate for the needs for the hot and humid environment. It is quite possible that, during the process of developing acclimatization as the New Orleans summer developed, these patients drank more and more water which became a habit to be maintained for a time regardless of need. Therefore, when they entered the comfortable environment they continued to drink large quantities of water by force of habit rather than for physiologic requirements. Furthermore, the large intake of water and the resultant diuresis inhibited any manifestations of the environmental effects of excretion of tritium and its biologic half-life. Since output of H_2O was determined by a very high intake, mechanisms for conservation of H_2O did not have to develop. Thus, total excretion of H^3, regardless of the proportion in sweat and in urine, was high and fairly constant (Figs. 1 to 3). Had H_2O intake been limited to minimal urinary needs in the neutral or comfortable environment, this intake would have been inadequate in the hot environment when H_2O was deviated into sweat for thermal regulation.

The experiments demonstrate the possibility of employing several radioactive isotopes simultaneously for physiologic and pharmacologic studies over prolonged periods of time in intact man. The tracers were readily separated with accurate counting. The findings with the simultaneous studies of several tracers were the same as obtained previously in studies of individual isotopes

and, therefore, increase the series for control subjects and for patients with chronic congestive heart failure. The various illustrations reveal the temporal qualitative and quantitative relationships of the 3 tracers used and interrelationships of physiologic adjustments involving water, chloride, and potassium.

SUMMARY AND CONCLUSIONS

Simultaneous studies of Cl^{36}, Rb^{86}, and H^3 under controlled metabolic conditions were made in both control subjects and in patients with congestive heart failure. The effect of a comfortable air-conditioned ward and of a hot and humid environment on kinetics and metabolism of electrolytes and water in both groups of subjects was studied. Even though there was little measurable effect of environmental temperature on the parameters observed in this study, there were rather distinct differences in the effect of the environment from a clinical standpoint. Possible reasons for the discrepancies between physiologic and clinical data have been discussed.

These experiments show the feasibility of long-term metabolic studies using a combination of isotopes to observe extracellular and intracellular electrolyte metabolism simultaneously with studies of turnover of body water. Additional data on the biologic decay rates of Rb^{86} and Cl^{36} have been presented along with data on the measurement of body water with tritium.

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STUDIES OF THE INFLUENCE OF COMFORTABLE AND HOT AND HUMID ENVIRONMENTS UPON THE RATE OF EXCRETION OF H^3, H_2O, Cl^{36}, Cl^{35}, Na^{23}, Rb^{86}, AND K^{39} IN CONTROL SUBJECTS AND PATIENTS WITH CONGESTIVE HEART FAILURE

G. E. Burch, M.D.,* and C. T. Ray, M.D.**
New Orleans, La.

STUDIES were initiated to investigate the influence of a hot and humid hospital environment of New Orleans during the summer upon certain phases of electrolyte and water metabolism in subjects with and without chronic congestive heart failure. Other aspects of these experiments have been described in a preceding paper.[1] The hot and humid environment produced definite changes in the urinary excretion of water and electrolytes associated with an increase in sweating in most of the subjects. Contrastingly, the comfortable air-conditioned ward revealed the influence of air conditioning upon renal and sweat gland function.

METHODS AND MATERIALS

Nine women were studied under controlled metabolic conditions in the two wards of Charity Hospital (Table I). One ward was air conditioned (mean temperature 74.4° F., range 80.2° maximum to 70.1° minimum) with a mean relative humidity of 73.4 per cent (range 90 to 60 per cent), while the environment of the other ward was hot and humid—the weather existing in New Orleans in July and August, 1956 (mean temperature 83.4° F., range 94.0° maximum to 69.0° minimum), with a mean relative humidity of 80.0 per cent (range 98 to 47 per cent). The maximum daily temperature was 90.0° F., or greater on 21 of the 26 days of this study. The average daily temperature recorded twice daily on the ward was 86.8° F. with a relative humidity of 62.0 per cent. The subjects in this study spent approximately equal time in both environments. One-half of the patients were in the comfortable environment first and, after approximately 2 weeks, were transferred to the other environment. At the onset of these studies 5 subjects received intravenously Cl^{36}, Rb^{86}, and H^3OH simultaneously.

Dietary intake of sodium, chloride, potassium, and water was recorded and the urinary excretion of these substances was measured. No measurements were made of the sweat or stools.

Blood was collected under oil each morning, and the plasma and whole blood were studied separately. The following measurements were made on the plasma: sodium and potassium by flame photometry, chloride by the method of Schales and Schales,[2] osmolarity by the Fiske osmometer, creatinine by the method of Haugen[3], Rb^{86} and Cl^{36} by

From the Department of Medicine, Tulane University School of Medicine, and Charity Hospital of Louisiana, New Orleans, La.

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*Henderson Professor and Chairman, Tulane University School of Medicine; Physician-in-Chief, Tulane Unit, Charity Hospital of Louisiana, New Orleans, La.

**Associate Professor of Medicine, Tulane University School of Medicine; Senior Visiting Physician, Tulane Unit, Charity Hospital of Louisiana, New Orleans, La.

end window Geiger-Müller counters,[4] and H^3 with a liquid scintillation counter.* After laking by freezing and thawing of an aliquot of whole blood, the sodium, potassium, Rb^{86}, Cl^{36}, and H^3 were measured.

The 24-hour urine specimens, collected from 8:00 A.M. to 8:00 A.M. with toluene as the preservative, were studied by the above methods for sodium, choride, potassium, Rb^{86}, Cl^{36}, H^3, osmolarity, and creatinine. Urinary volume, specific gravity, and pH were also determined.

Separate radioassay of Rb^{86} and Cl^{36} was accomplished by counting soon after collection of the samples and recounting the preparations 200 days later after Rb^{86} had undergone physical decay. Radioassay of tritium was done on a water distillate of the biologic fluids which were distilled at low temperatures so that Cl^{36} did not enter the distillate.

Dietary intake of sodium, potassium, chloride, and calories was kept relatively constant, but water was allowed as desired. The subjects received various drugs and therapeutic measures as indicated by the clinical state (Table I).

TABLE I. CLINICAL DATA ON THE NINE SUBJECTS STUDIED

SUBJECT NO.	AGE (YR.)	DIAGNOSIS	DOSE OF ISOTOPES			TREATMENT
			Cl^{36} (MC.)	Rb^{86} (MC.)	H^3 (MC.)	
1	46	Control; respiratory infection, convalescent	0.026	0.631	3.749	None
2	50	Control; tuberculous arthritis	0.027	0.663	4.104	Streptomycin, sodium para-aminosalicylate, body and leg cast
3	52	R.H.D., C.H.F.	0.020	0.481	2.993	Digitalis, mercurial diuretics
4	52	A.S.H.D., C.H.F., H.H.D.	0.027	0.662	3.780	Digitalis, mercurial diuretics
5*	65	A.S.H.D., empyema, CH.F., pneumothorax	0.026	0.642	3.852	Digitalis, thoracentesis, penicillin
6	23	Control; sarcoidosis				Prednisone, 30 mg. daily
7	32	Control; lung abscess, convalescent				Penicillin, Achromycin
8	22	R.H.D., C.H.F.				Digitalis, prednisone, 30 mg. daily
9	56	A.S.H.D., mild angina, C.H.F.				Digitalis, mercurial diuretics

Key to abbreviations: R.H.D. = rheumatic heart disease; C.H.F. = congestive heart disease; A.S.H.D. = arteriosclerotic heart disease; H.H.D. = hypertensive heart disease.

*Incomplete urine collections.

RESULTS

The results are summarized in Figs. 1 to 8 and in Tables II, III, and IV. The data are presented as follows: clearance rates; daily excretion of chloride and potassium as a per cent of calculated mass; and osmolar and free water excretion, glomerular filtration, and renal tubular reabsorption.

*The authors wish to express their appreciation to Drs. Leon Jacobsen and George Okita of the University of Chicago School of Medicine for the use of their laboratory facilities for counting the tritium samples.

Urinary Clearance Rates.—Average daily clearance rates were determined for Cl^{36}, Cl^{35}, Na^{23}, Rb^{86}, K^{39}, and H^3 in 4 subjects (Figs. 1 and 2) and for Cl^{35}, Na^{23}, and K^{39} in 4 additional subjects (Figs. 3 and 4).

Rb^{86} and K^{39} clearances: The qualitative and quantitative similarities of the clearance rates of Rb^{86} and K^{39} are evident. Variations exist in rate of clearance of potassium without an equal quantitative change for Rb^{86}, but qualitative differences were few (12 points of qualitative directional differences in 4 subjects) (Figs. 1 and 2). That potassium was excreted preferentially over Rb^{86} is apparent from these graphs, but Rb^{86} traced K^{39} reasonably well, the correlation coefficient being +0.816. This is approximately the same relationship for excretory rates previously described.[5]

The average daily rates of clearance of potassium varied widely among the subjects and in the same subject from time to time, the minimal rate being about 4 ml. of plasma cleared per minute and the maximal rate about 18 ml. per minute. The magnitude of variations within each individual subject was almost as great as the variations among the subjects. There was no apparent effect of the clinical state of congestive heart failure or of the two environmental conditions on the average rate of clearance of K^{39} or Rb^{86}. Subject 1 had a very high rate of clearance on the first day in the cool environment. The intake of potassium was higher in Subject 8 who was given added KCl along with prednisone. The average rates of clearance of K^{39} in this subject and in Subject 6, who likewise received prednisone, were greater than in the other subjects (Figs. 1 to 4).

Administration of a mercurial diuretic had a variable effect on the rates of clearance of K^{39} and Rb^{86} (Figs. 2 and 4).

Comment: The quantitative differences in rates of clearance of K^{39} and Rb^{86} reflect a difference between these two isotopes which was greater for the kidney than for any other organ studied to date.[5, 6] The reason for this difference in excretory rates is not apparent, but may be related to differences in atomic masses in spite of about equal sizes for the hydrated ions.

The absolute values of clearance of K^{39} among the various subjects, as expected, reflected the variations in daily intake of potassium, which varied from 52 to 79 mEq. per day in 7 subjects and was as high as 118 mEq. per day in the subject who received added KCl. However, the day-to-day intake of potassium varied relatively little for any given subject. Such small daily variations in intake in any subject were insufficient to explain the marked differences in daily rates of clearance of potassium. Furthermore, the change in intake and rate of clearance were not always directionally related. Interestingly, the clearance rates were not influenced by the environments employed. The effects of prednisone on excretion and balance of potassium are shown by the negative balance in Subject 6. Since nonurinary losses of K^{39} were not measured, the exact deficit of potassium in this subject was not measurable. The progressive increase in cumulative balance of potassium in all other subjects reflected, in large part, nonurinary loss.

Tritium clearance: The rates of clearance of H^3 for 4 subjects are summarized in Figs. 1 and 2. In Subjects 1 and 3, the rates of clearance were slightly higher in the cool environment. The various "isotope effects" due to greater atomic mass and other physical phenomena which have been ascribed to H^3 as a tracer of H^1 were not reflected in the urinary clearance rates as indicated by the correlation coefficient of clearance of H^3 to water clearance of +0.969.

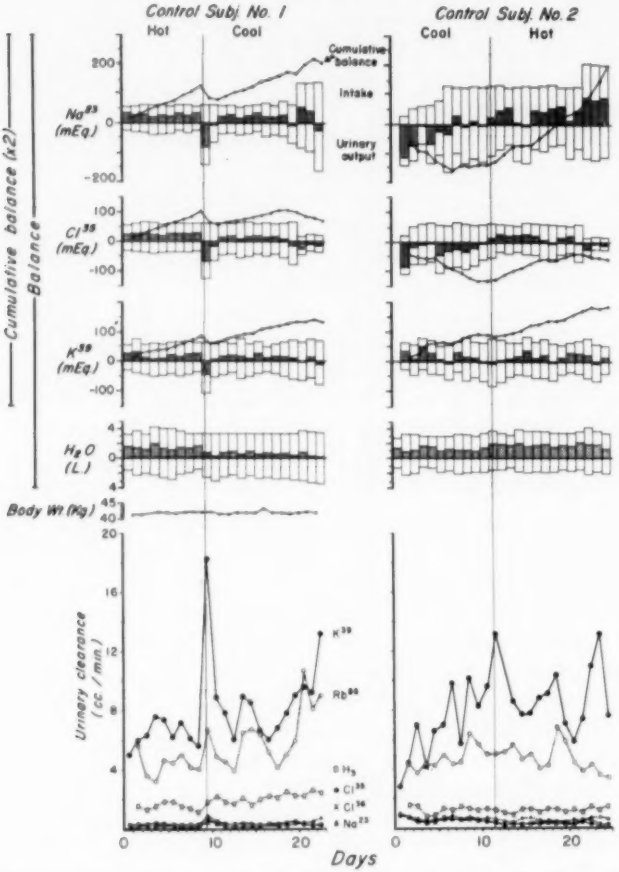

Fig. 1.—Time course of urinary clearance of K^{39}, Rb^{86}, H^3, Cl^{35}, Cl^{36}, and Na^{23} in 2 control subjects. In this and all subsequent figures the balance data is shown in the upper part of the figure. The ordinate values for cumulative balance are twice those shown for daily intake, urinary output, and balance. The values for average daily urinary clearances of the radioisotopes and nontracer elements are shown in the lower part of the figure. Body weights were not obtainable on Control Subject 2 since she was in a body and leg cast.

When mercurial diuretics and spontaneous factors increased the rates of clearance of Na^{23} and Cl^{35}, clearance of H^3 increased. The changes in these clearances varied concordantly yet they differed quantitatively. The clearance rates of H^3 varied from 0.5 ml. to 2.7 ml. per minute in Subject 3 as a

result of mercurial diuresis. The other subject with congestive heart failure (Subject 4) had slightly lower rates of clearance of H^3 than the control subjects (Subjects 1 and 2).

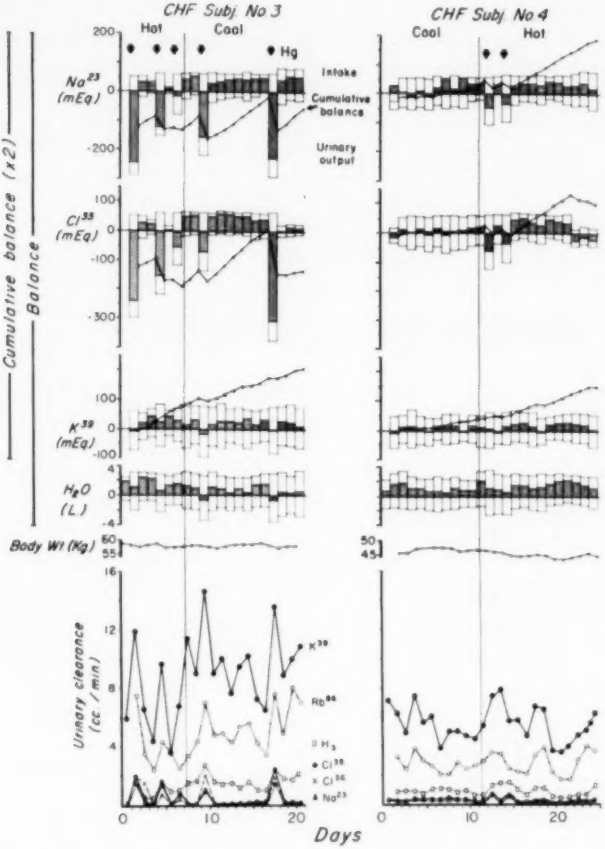

Fig. 2.—Time course of urinary clearance of K^{39}, Rb^{86}, H^3, Cl^{35}, Cl^{36}, and Na^{23} in 2 subjects with congestive heart failure. See legend for Fig. 1.

Comment: The rate of urinary clearance of H^3 varied directly with water intake and inversely with the rate of nonurinary water loss. Since these patients (Subjects 1 to 4) had little change in weight, the rate of clearance of H^3 reflected the water drinking habits of the individual. The correlation coefficient of +0.969 between the rates of clearance of H^3 and the rates of clearance of plasma water indicated the absence of skimming, isotope effects due to differences in mass of H^1 and H^3, and the excretion of significant amounts of H^3 in molecular combinations other than in water.

The relationship of rates of clearance of H^3 to that of Cl^{36}, Na^{23}, and K^{39} indicates that the subjects who were allowed water as desired drank sufficient

water to maintain approximately the same rate of free water excretion for both environments.

Cl^{36}, Cl^{35}, and Na^{24} clearances: The rates of clearance of Cl^{35} and of Cl^{36} had a correlation coefficient of +0.949 (Figs. 1 and 2). This value should be unity, but the low levels of counts of Cl^{36} in some specimens of urine increased the counting error. The time course of the relationship of the rates of clearance of Cl^{36} to Cl^{35} is shown for 4 subjects in Figs. 1 and 2.

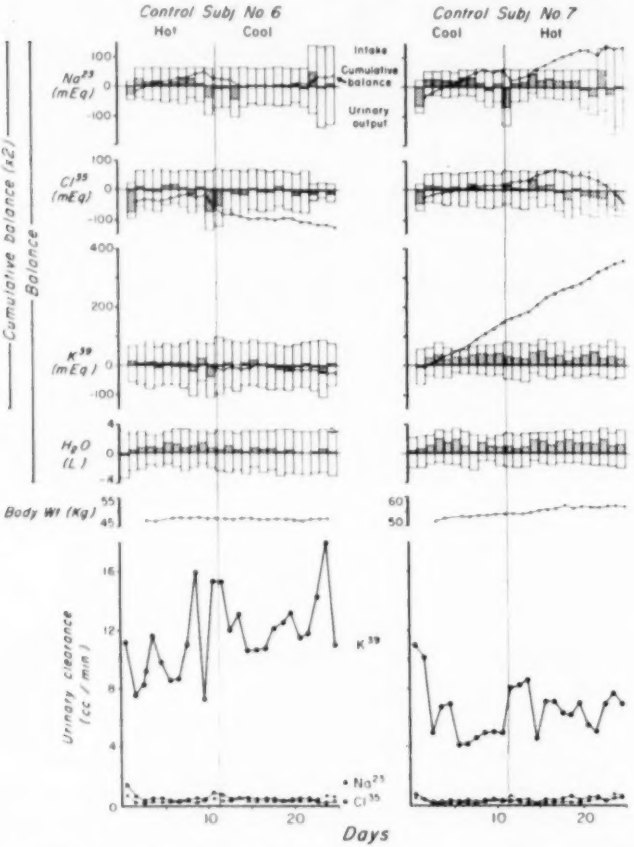

Fig. 3.—Time course of urinary clearance of K^{39}, Cl^{35}, and Na^{23} in 2 control subjects. See legend for Fig. 1.

The rates of clearance of Cl^{35} and Na^{23} were much lower than those for Rb^{86}, H^3, and K^{39} (Figs. 1 to 4 and Table II). The relationship of these rates of clearance to sodium and chloride balance are summarized in Figs. 1 to 4. The rate of clearance of sodium was approximately 75 per cent that of chloride in Subjects 6, 7, and 9; however, in Subject 2, the clearance of sodium was slightly

greater than that for chloride, and in Subject 8 the clearance of Cl was considerably greater than that for Na, these differences among subjects being related to intake of these electrolytes. There was no consistent effect of environment on these rates of clearance. The effects of mercurial diuresis on these subjects made the effect of environment difficult to evaluate.

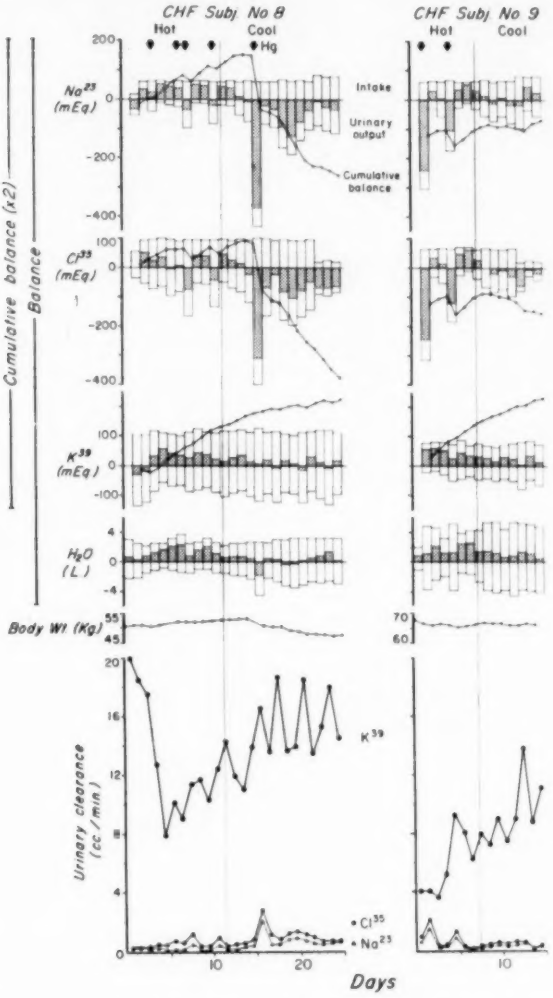

Fig. 4.—Time course of urinary clearance of K^{39}, Cl^{35}, and Na^{23} in 2 subjects with congestive heart failure. See legend for Fig. 1.

Comment: The well-known effects of dietary intake of sodium and chloride on the respective clearance rates are evident in the illustrations. In none of

the subjects were there any significant changes in the concentration of chloride and sodium in the serum.

TABLE II. THE EFFECT OF ENVIRONMENT ON URINARY CLEARANCE RATES

SUBJECT NO.	PERIOD STUDIED	AVERAGE CLEARANCES (ML./MIN.)						AVERAGE DAILY URINE VOLUME (ML.)
		K^{39}	Rb^{86}	Cl^{36}	Cl^{35}	Na^{23}	H^3	
1	Entire study	8.13	5.55	0.30	0.32	0.26	1.87	2,592
	Hot	6.55	4.32	0.23	0.26	0.16	1.46	2,249
	Cold	9.04	6.26	0.34	0.36	0.32	2.09	2,787
2	Entire study	8.42	4.73	0.36	0.37	0.55	1.24	1,790
	Hot	9.23	4.72	0.25	0.27	0.50	1.23	1,780
	Cold	7.27	4.76	0.52	0.52	0.61	1.27	1,800
3	Entire study	9.08	4.94	0.46	0.44	0.32	1.44	2,009
	Hot	7.15	3.97	0.75	0.73	0.44	0.98	1,391
	Cold	9.91	5.36	0.34	0.32	0.28	1.64	2,273
4	Entire study	5.53	2.86	0.29	0.29	0.22	0.92	1,595
	Hot	5.60	2.93	0.29	0.27	0.21	0.94	1,556
	Cold	5.42	2.75	0.29	0.31	0.24	0.90	1,651
6	Entire study	11.72			0.45	0.34		1,640
	Hot	10.45			0.47	0.26		1,348
	Cold	12.64			0.43	0.39		1,849
7	Entire study	6.42			0.38	0.28		1,591
	Hot	6.85			0.42	0.34		1,612
	Cold	5.83			0.34	0.21		1,561
8	Entire study	13.71			0.78	0.41		2,164
	Hot	12.16			0.54	0.18		1,364
	Cold	14.81			0.95	0.58		2,735
9	Entire study	7.94			0.54	0.38		3,355
	Hot	6.12			0.70	0.50		2,574
	Cold	9.30			0.42	0.30		3,940

Some of the effects of the environment observed for Control Subjects 1 and 2 might be expected on the basis of loss of sodium and chloride in sweat because of the hot and humid environment. The cardiovascular stress of temperature regulation has been indicated previously.[7] Among the 4 patients with congestive heart failure there was only one instance where the clinical state of failure was influenced by the environment if this is reflected in excretory rates. The spontaneous diuresis which developed in Subject 8 on entering the cool and comfortable environment may have been the result of relief from the cardiovascular burden of heat regulation. Like lessening the load of muscular exercise by bed rest, lessening the burden of body temperature regulation may have resulted in the diuresis.

Urinary Excretion of Cl^{35} and K^{39} as a Per Cent of Calculated Mass.—The mass of Cl^{35} and of K^{39} was calculated from tracer dilution in 4 patients and daily excretion was expressed as a per cent of the "calculated" masses (Table III). The excretion of chloride was expressed as a per cent of average daily M_t for the entire study whereas excretion of K^{39} was expressed as a per cent of the estimated K^{39} mass during the period of days when this value was constant.

Since there was a rise in calculated M_t for potassium toward the end of the experiment as a result of cellular lags in exchange of Rb^{86} with plasma, the period of relatively constant M_t was selected for these calculations.

TABLE III. EFFECT OF ENVIRONMENT ON URINARY EXCRETION OF CHLORIDE AND POTASSIUM

SUBJECT NO.	DAILY EXCRETION AS A PER CENT OF THE AVERAGE CALCULATED MASS					
	Cl^{35}			K^{39}*		
	ENTIRE STUDY	HOT	COLD	ENTIRE STUDY	HOT	COLD
1	2.35	1.79	2.73	1.40	1.17	1.55
2	3.36	2.31	4.72	1.67	1.81	1.50
3†	4.29	6.48	3.19	1.96	1.48	2.20
4†	1.78	1.67	1.93	1.17	1.15	1.20

*Calculated from data on Rb^{86}.
†Congestive heart failure.

Chloride: The excretion of Cl^{35} as a per cent of calculated M_t for the 2 control subjects differed considerably (Table III). Subject 1 excreted about 2 per cent of her Cl^{35} mass per day with a peak rate noted on the day of transfer into the cool environment. Subject 2 excreted more chloride during the exposure to the cool environment (average about 4 per cent) than while she was in the hot and humid one (average 2.5 per cent). Subject 3 with congestive heart failure excreted slightly less than 1 per cent of M_t on the days when she did not receive mercurial diuretics. The profound effect of mercurials on excretion of chloride was demonstrated in this subject who excreted about 1/4 of the chloride calculated to be in her body in one day of mercurial diuresis. The other patient with congestive heart failure (Subject 4) excreted about 2 per cent of M_t daily while in the cool environment. In the hot and humid environment there was a decrease in excretory rate on the days when she did not receive a mercurial diuretic.

Comment: During periods of zero balance of any electrolyte, the urinary excretion should represent the same per cent of calculated M_t as does intake, provided there are no important extraurinary losses of these substances. The dietary intake of chloride was 2.7, 3.0, 3.6, and 2.1 per cent of calculated M_t for Subjects 1 to 4, respectively.

Potassium: The time course of daily excretion of potassium expressed as a per cent of the average calculated M_t did not bear any directional relation with that of chloride except on the days of mercurial diuresis in Subjects 3 and 4 (Fig. 2) and in Subject 1 on her first day in the cool environment. The average per cent of M_t excreted per day was 1.2, 1.5, 1.9, and 1.1 for Subjects 1 to 4, respectively, the range being 0.6 to 3.4 per cent. The average dietary intake of potassium was 1.7, 2.3, 2.8, and 1.5 per cent of average calculated M_t for Subjects 1 to 4, respectively. There was no effect of change in the environment on the rates of excretion of potassium except in Subject 1 (Fig. 1) who had a marked diuresis of K^{39} on being transferred from the hot and humid to the comfortable environment.

TABLE IV. THE INFLUENCE OF ENVIRONMENT ON THE URINARY EXCRETION OF SOLUTE

SUBJECT NO.	mOsm. FILTERED (mOsm./min.)			mOsm. EXCRETED (mOsm./min.)			mOsm. REABSORBED (mOsm./min.)			ENDOGENOUS CREATININE CLEARANCE (ml./min.)		
	ENTIRE STUDY	HOT	COLD	ENTIRE STUDY	HOT	COLD	ENTIRE STUDY	HOT	COLD	ENTIRE STUDY	HOT	COLD
1	21.93	21.19	22.40	0.295	0.266	0.313	21.63	20.93	22.09	79.10	77.18	80.34
2	19.47	17.81	21.58	0.338	0.343	0.331	19.13	17.47	21.25	67.51	64.42	78.58
3*	21.03	17.02	23.03	0.336	0.310	0.348	20.69	16.71	22.69	74.63	60.33	81.78
4*	16.13	16.69	15.43	0.299	0.304	0.293	15.83	16.38	15.13	59.04	61.34	56.13
6	31.49	30.37	32.37	0.523	0.554	0.506	30.96	29.81	31.86	113.46	109.53	116.54
7	24.97	24.86	25.11	0.260	0.223	0.299	24.70	24.56	24.88	92.11	91.60	92.75
8*	24.22	24.55	23.96	0.497	0.423	0.555	23.72	24.13	23.40	87.14	89.12	85.59
9*	31.10	31.47	30.78	0.436	0.410	0.460	30.66	31.06	30.32	109.18	110.31	108.19
Average	23.79	23.00	24.33	0.373	0.354	0.388	23.42	22.63	23.95	85.27	82.98	87.49

*Congestive heart failure.

Osmolar Excretion, Glomerular Filtration, and Tubular Reabsorption.—The data of plasma and urine osmolarity, osmolar, free and total water clearance rates, milliosmolar glomerular filtration rates, and endogenous creatinine clearance rates are summarized in Figs. 5, 6, 7, and 8 and Table IV.

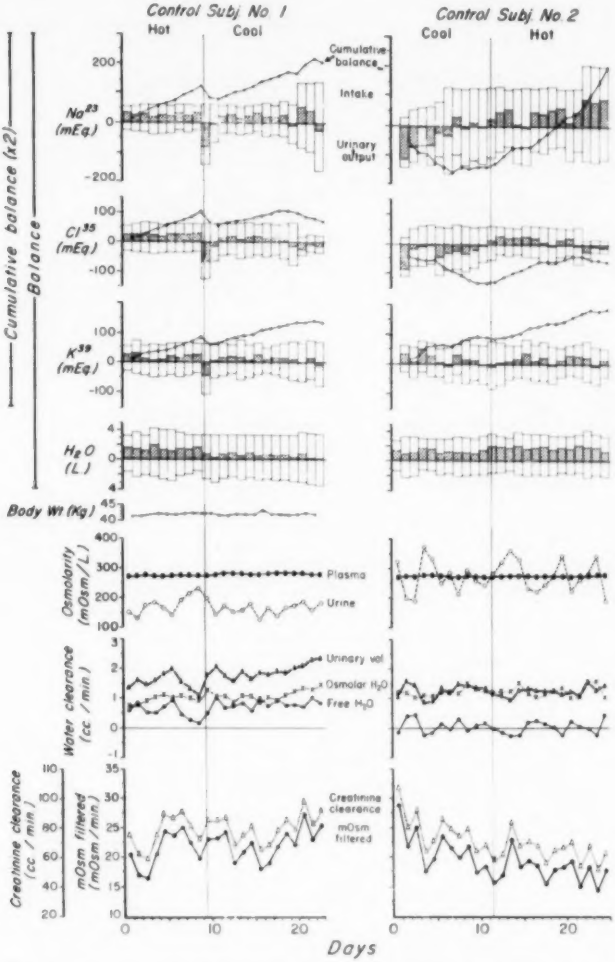

Fig. 5.—Time course of urinary excretion of water in 2 control subjects. The balance data is presented as in Fig. 1. In addition, the osmolarity of plasma and urine, urinary volume, osmolar and free water clearance, and endogenous creatinine clearance and milliosmoles filtered are shown in the lower part of the charts.

Plasma osmolarity changed very little throughout the entire period of observation. Urinary osmolarity, on the other hand, showed considerable variation among the subjects as well as in the same subject in the two environmental

conditions. In Control Subjects 1 and 2 (Fig. 5) there was no difference in osmolarity of the urine in the two environments, while in the remaining 6 subjects the urine osmolarity was higher when they were in the hot and humid environment.

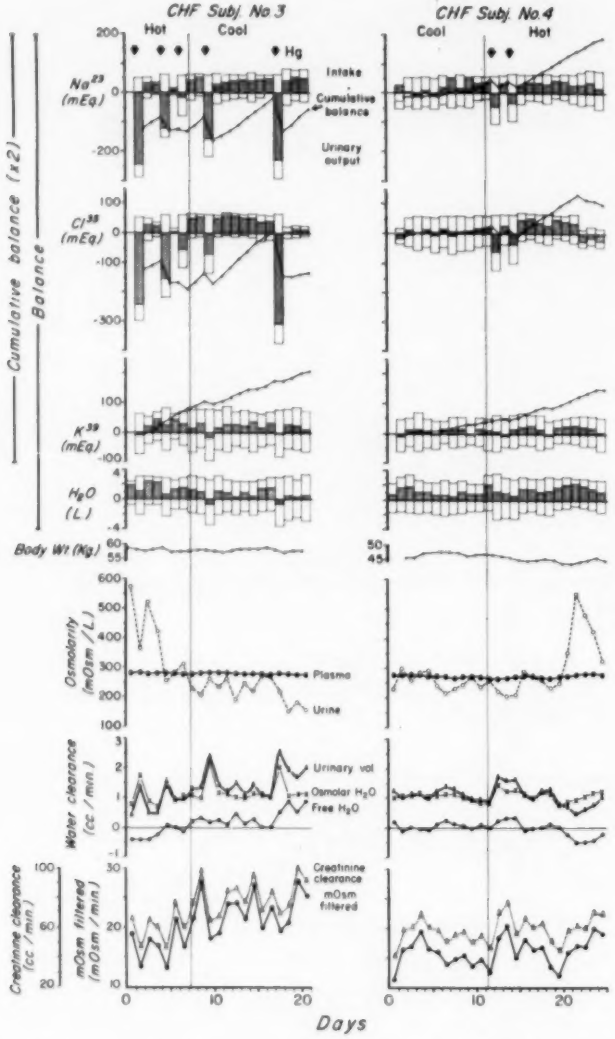

Fig. 6.—Time course of urinary excretion of water in 2 subjects with congestive heart failure. See legend for Fig. 5.

The time-course curves of average daily free water, osmolar water, and total water clearance rates expressed in milliliters per minute show considerable

variation among the 8 subjects whose urinary collections were complete. Subjects 1 and 9 (Figs. 5 and 8) had free water excretion throughout the entire observation.

The effect of a mercurial diuretic on free water excretion was variable. The response to mercurials was not influenced by administration of a diuretic during periods when there was either positive or negative free water clearances.

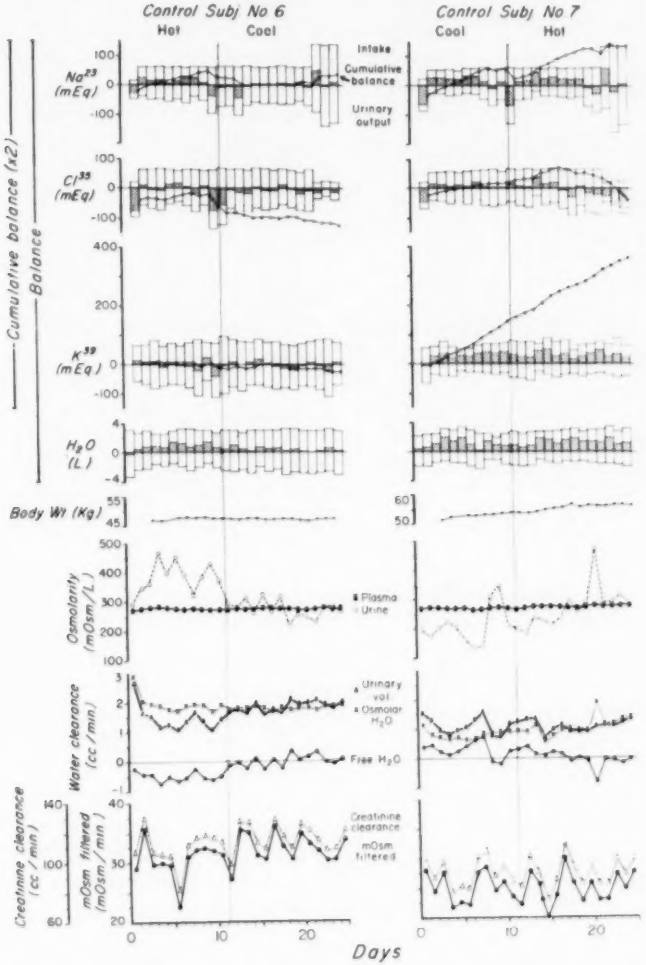

Fig. 7.—Time course of urinary excretion of water in 2 control subjects. See legend for Fig. 5.

Comment: The relative constancy of plasma osmolarity throughout these studies was to be expected with adequate electrolyte and water intake. One

severely ill subject in this study, not shown in these figures, became dehydrated in the hot and humid environment and the plasma osmolarity rose by 20 mOsm. per liter.

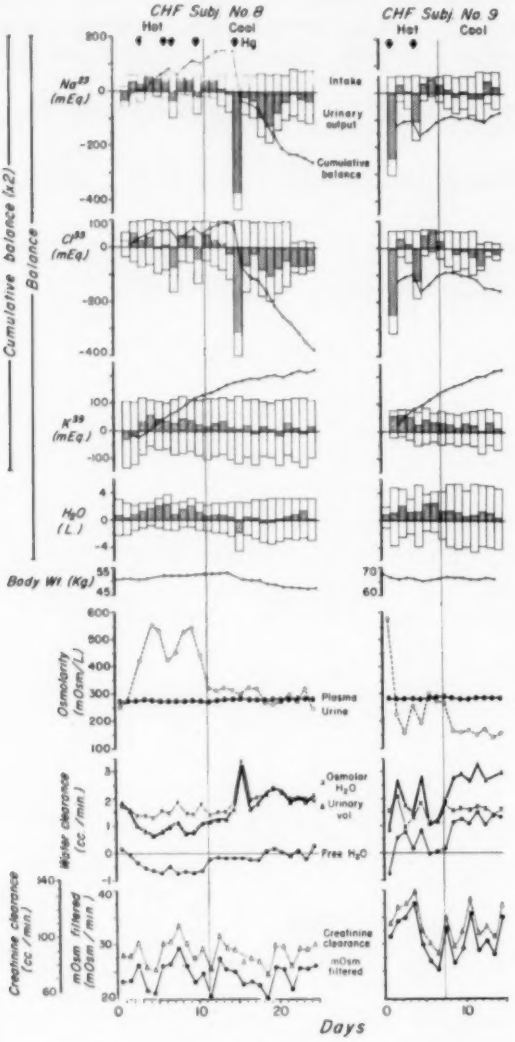

Fig. 8.—Time course of urinary excretion of water in 2 subjects with congestive heart failure. See legend for Fig. 5.

The osmolarity of the urine in these subjects would be expected to vary according to the relative amounts of electrolyte and water intake which determines

the amount to be excreted to maintain balance. With sweating, the osmolarity of the urine would be expected to be greater in the hot environment than in the cool one.

Free water, and thus total water excretion, reflected the difference between intake and nonurinary losses by respiratory tract and skin. Since these subjects were allowed water as desired, free water excretion reflected drinking habits, which kept two of the subjects in a state of constant water diuresis even in the hot and humid environment. The unusual drinking of water by Subject 9 when in the cool environment resulted in urinary volumes of approximately 4,000 ml. daily (Fig. 8). The remainder of the subjects had a daily water intake which resulted in a urinary osmolarity approximately equal to the plasma so that there was little free water clearance. The urine in Control Subject 6 and Subject 8 with heart failure was hyperosmolar to plasma with a $T^c_{H_2O}$ value of about 0.5 ml. per minute while in the hot and humid environment. Some of the subjects with congestive heart failure had urine hyperosmolar to plasma before therapy for their failure had begun. The two subjects on prednisone had higher osmotic loads for urinary excretion than the other subjects as evidenced by the prolonged periods of high osmolar water excretion.

Glomerular filtration rate: The average daily glomerular filtration rate was measured by endogenous creatinine clearances. The time courses of glomerular filtration rates are shown in Figs. 5, 6, 7, and 8 and Table IV. There were definite daily variations in the same subject. These variations were not influenced by osmolar load, the environment, or mercurial or spontaneous diuresis nor were they related to magnitude of osmolar, free, or total water clearance rates. The average creatinine clearance was slightly higher in the cool environment (86.49 ml./min.) than in the hot and humid one (82.98 ml./min.). There were periods when the reverse was true in 3 of the 8 subjects. The rates of creatinine clearance were as large in the subjects with congestive heart failure as in the normal control ones. In addition, the subjects with heart failure showed less change in glomerular filtration rate in the two environments than did the controls.

The average rate of glomerular filtration of mOsm. per minute summarized in Figs. 5 to 8 and Table IV were derived from the creatinine clearance and the osmolarity of the plasma for the same time. The milliosmolar reabsorption was about 98.5 per cent of that excreted throughout the period of study. There was a slight increase in the milliosmoles excreted in the cool environment over the hot and humid one, but the difference amounted to only 49 mOsm. per day, an amount well within the expected loss by sweating. In each subject, the milliosmolar excretion tended to be fairly constant with larger amounts excreted during mercurial and spontaneous diuresis.

Comment: Of the 4 subjects with congestive heart failure, 3 were moderately severe. However, since their rates of glomerular filtration were as high as those of the control subjects, inadequate glomerular filtration was not involved in the production of the clinical syndrome of congestive heart failure in these subjects.

The daily variability of glomerular filtration rate in both control subjects and in the subjects with heart failure was quite impressive. This has been true

for all substances measured in the urine in this laboratory.[5, 8, 9, 10] Moment-to-moment variations of this function were not obtained in these experiments as was done in other studies in the past. However, large day-to-day variations were noted even though the subjects were at bed rest and in a steady metabolic state. These findings indicate further the need for multiple and prolonged observations of renal functions in order to establish the range of normal and to assess the effects of various procedures on such renal phenomena.

The relative independence of the osmolar load excreted from osmolar filtration is evident in these data. Adjustments to the needs of balance of both water and electrolytes over a wide range of filtration rates, and under the circumstances of two quite different environments, must have been primarily tubular reactions.

DISCUSSION

These studies revealed several interesting urinary excretory phenomena. For example, a hot and humid summer environment produced in most of the subjects a significant increase in extrarenal losses of water and electrolytes even though the subjects were at bed rest. In addition, there was an increase in osmolarity of the urine with a tendency to negative free water clearance. An unusual voluntary intake of water developed in some people acclimatized to a hot and humid summer and continued while they were in the air-conditioned ward where extra requirements for water were not necessary. The habit of drinking large quantities of water appeared to us to be a manifestation of acclimatizing adjustments made by some people of Louisiana to the heat and humidity of the summer. Probably with a longer period of residence in the cool environment the intake would have declined to the usual levels. Furthermore, the high intake, although adequate for the hot and humid environment and assuring essentially an average normal urinary state, resulted in a dilute urine of large volume when continued in the cool environment.

These experiments revealed the marked fluctuations in urinary clearance and excretion of Cl^{36}, Cl^{35}, Na^{23}, Rb^{86}, K^{39}, H^3, and H_2O when intake of these substances and physical activities were relatively constant. These marked daily variations support further similar previous observations[5, 8-10] of marked fluctuations over shorter periods of time as well as for daily intervals. In view of such variations, it is necessary to exercise caution when observing renal function and urinary excretion with only a few such measurements.

The daily endogenous creatinine clearances recorded for 26 consecutive days revealed no significant differences between the time course and magnitude of the rates of glomerular creatinine clearance of the subjects with or without chronic congestive heart failure. The patients with heart failure had both remissions and exacerbations in their failure during the observation period. It was evident from these experiments that any variations in renal function associated with the congestive failure were primarily tubular. Furthermore, it would likewise appear that unless congestive failure is severe, glomerular filtration remains within normal limits, at least for these subjects with moderately severe chronic congestive heart failure.

These experiments reveal the facility with which renal function may be followed continuously and simultaneously with different radioactive isotopes. Although only 3 were employed in this study, a larger number could be used equally well by taking advantage of differences in rates of physical decay and energy and spectra of radiation. Volatilization[11] and other special physical and chemical characteristics of the element may increase further the number which may be employed. There are many advantages in observing several different tracers simultaneously in a given animal rather than tracing each separately.

Again, these experiments confirm physiologic data previously described in man[5, 10, 12] with and without chronic congestive heart failure for Cl^{36}, Cl^{35}, Na^{22}, Na^{23}, Rb^{86}, and K^{39} and add the simultaneously observed renal excretion of H^3OH. These experiments add support to already existing data, thus increasing the validity of the findings for man as a whole.

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THE PRESERVATION OF BLOOD FOR TRANSFUSION

III. Mechanism of Action of Containers on Red Blood Cells

Max M. Strumia, M.D., Louise S. Colwell, A.B., and Ann Dugan,
Bryn Mawr, Pa.

With the Technical Assistance of Jean Bentz, A.A.

It has been shown that red cells collected and stored as whole blood are differently affected by the nature of the containers, as evidenced by in vitro tests and posttransfusion survival studies.[1, 2] The purpose of this investigation is to gain information on the mechanism of this effect.

We may consider the effect of containers on the red cells as possibly due to: (1) substances leached from the containers; (2) the liquid-air interface; (3) the water repellence and other physical properties. In the experimental work here presented these factors were analyzed individually.

MATERIALS AND METHODS

For the study of the effect of leached substances, donors were bled from the same venipuncture in 2 to 4 separate containers. The blood specimens were collected in ACD solution, formula NIH-A, at room temperature; the containers with the blood were then placed in an ice water bath for one-half hour and then stored at 5° C. One of the containers for each donor was always a standard borosilicate glass bottle, as control; a similar bottle contained strips of the plastic in amounts equivalent to the surface of a bag normally in contact with the blood. Whole plastic bags were also used, as controls, whenever commercially available (plastics C and X).

In addition to testing the plastic material actually used in bags available commercially for storage of blood, samples of plastic material were obtained to test the effect of plasticizers, stabilizers, and mold-releasing agents.

Plastic material EA-2158, EA-2159, and EA-2160 consisted of samples of laboratory-prepared polyvinyl chloride sheeting* of the following composition:

	EA-2158	EA-2159	EA-2160
Geon 101 EP (polyvinyl chloride)	62	62	62
Plexol 201-W (distilled DOS)	38	—	—
Monoplex DOS	—	38	38
Calcium stearate	—	—	1
Harshaw's 9V-1	—	—	2

Plastic sample EA-2158 is made of polyvinyl chloride plasticized with a distilled grade of dioctyl sebacate: this represents a higher quality than the Monoplex DOS, a standard grade of plasticizer used in material EA-2159. In sample EA-2160, calcium stearate is added as a mold-releasing agent and Harshaw's 9V-1 as a stabilizer. This material is a zinc compound

From The John S. Sharpe Research Foundation and the Laboratory of Clinical Pathology of The Bryn Mawr Hospital, Bryn Mawr, Pa.

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*Rohm & Haas, Philadelphia, Pa.

mixed or associated with alkyl aryl phosphite. The zinc content is 2.7 per cent. Zinc as a stabilizer is commonly used when low toxicity is one of the properties desired and is the only metal present in any appreciable quantity in this plastic. Glass bottles containing the ACD solution and the plastic strips were sterilized with steam for 30 minutes at 121° C. The method for the measurement of red cells survival by tagging with Cr^{51} and for the study of properties in vitro are described in a previous publication.[3]

For the study of the liquid-air interface, two plastic bags containing 75 ml. of ACD solution were used. With sterile technique, 37 ml. of solution was removed from each bag; in one, approximately 250 ml. of filtered air was introduced. A donor was bled of 500 ml. of blood, 250 ml. being introduced directly in each of the two bags. Blood was stored at 5° C. for 3 weeks.

For the study of the effect of immediate and prolonged contact blood was collected in containers of plastic, glass, and silicone-coated glass.* Blood was either cooled and stored in the same container or transferred before cooling to a container of a different nature as indicated in each experiment.

RESULTS

Effect of Substances Leached From the Containers.—The first series of experimental studies was directed to investigate the effect on red cells of substances possibly leached from the plastic. Table I shows the effect of the various plastics on the red cells, as determined by in vitro studies. It will be noted that the addition of plastic X, obtained from commercially available bags, and, to a lesser extent, of plastic EA-2158 is accompanied by an increase in the amount of free plasma hemoglobin. A similar effect is noted when blood is stored in a container made with plastic X. Hemolysis in 0.63 per cent saline solution appears to progress somewhat more rapidly when plastic X is added.

It is noted that damage to red cells, evidenced by increased free plasma hemoglobin, is exerted by the plastic material with or without the presence of glass surfaces. The high concentration of free plasma hemoglobin occurring when blood is collected in unsuitable plastic has been noted previously.[2] Plastic C added to a glass bottle does not increase significantly the concentration of free hemoglobin. All other data reveal no significant differences.

In subsequent experiments posttransfusion survival studies of red cells tagged with Cr^{51} were carried out simultaneously with the in vitro studies. Some of the plastic materials used in the first experiment were added to glass containers, and for each of 9 units of blood an autotransfusion and a transfusion in a compatible recipient were done, after a period of storage of 21 days.

In vitro studies showed variations in the plasma hemoglobin and in the adenosinetriphosphate (ATP) content, suggesting that the addition of plastic EA-2158 caused some damage to the red cells. Much clearer evidence of damage is shown by the results of posttranfusion survival of the same units of blood (Fig. 1). Normal posttransfusion survival was noted with the addition of plastic EA-2159. Similar results were obtained with plastic EA-2160, except for a single unit of blood. The average survival, however, of patients transfused with the units of blood to which plastic EA-2158 was added is definitely shorter

*All glass containers were of borosilicate and obtained from the Wheaton Glass Company, Vineland, N. J. Silicone 1107 was employed as obtained from Dow-Corning Corporation, Midland, Mich. Emulsion 35 A, obtained from the same corporation, was also used.

TABLE I

BLOOD UNIT	CONTAINER USED	PLASMA HEMOGLOBIN (MG./100 ML.)				PER CENT HEMOLYSIS IN 0.63% NaCl				ATP (MG./100 ML. OF RED CELLS)				DEXTROSE CONSUMPTION (MG./DAY/ML./OF RED CELLS)	LACTIC ACID PRODUCTION (MG./DAY/ML./OF RED CELLS)
		3*	4	5	6	3	4	5	6	3	4	5	6	6	6
372	Glass bottle Control	65	141	231	380	4	5	9	15	6.59	7.11	--	5.91	0.107	0.104
	Glass bottle Plastic C	104	133	226	355	4	5	6	9	5.58	7.38	--	4.17	0.108	0.096
	Plastic bag C	63	99	185	312	3	4	7	9	6.87	8.16	--	4.53	0.129	0.125
374	Glass bottle Control	151	208	337	370	6	9	12	22	6.24	4.95	3.72	3.78	0.086	0.080
	Glass bottle Plastic X	232	440	680	1140	7	9	15	24	7.78	6.29	3.60	--	0.069	0.069
	Plastic bag X	158	369	810	1060	10	13	22	28	5.54	4.83	4.95	0.3	0.079	0.086
373	Glass bottle Control	124	161	297	652	3	7	15	21	8.28	4.2	2.85	2.21	0.095	0.086
	Glass bottle Plastic EA-2158	145	192	290	484	4	7	11	21	7.71	4.11	3.66	3.21	0.108	0.078
379	Glass bottle Control	72	102	159	400	4	2	8	13	7.5	6.69	2.97	2.76	0.076	0.102
	Glass bottle Plastic EA-2158	116	150	265	355	4	2	7	9	7.32	3.81	3.93	3.77	0.070	0.103
	Glass bottle Plastic EA-2159	88	140	234	300	4	3	7	11	6.80	5.58	3.66	3.75	0.092	0.106
	Glass bottle Plastic EA-2160	50	91	146	229	4	2	5	8	9.02	4.59	3.93	3.26	0.083	0.100

*Numbers in subcolumns indicate weeks of storage.

For each unit of blood, designated by a number in the left-hand column, the results obtained with plastics must be evaluated by comparison of the data observed with the plain control glass bottles at various periods of storage, from 3 to 6 weeks.

than the average span observed for the units of blood stored in bottles to which other plastics were added. This evidence again suggests that some substance with a toxic effect on the red cells has been leached out of plastic EA-2158. The damaging effect of some commercially available plastic bags on red cells has been previously reported.[1, 2]

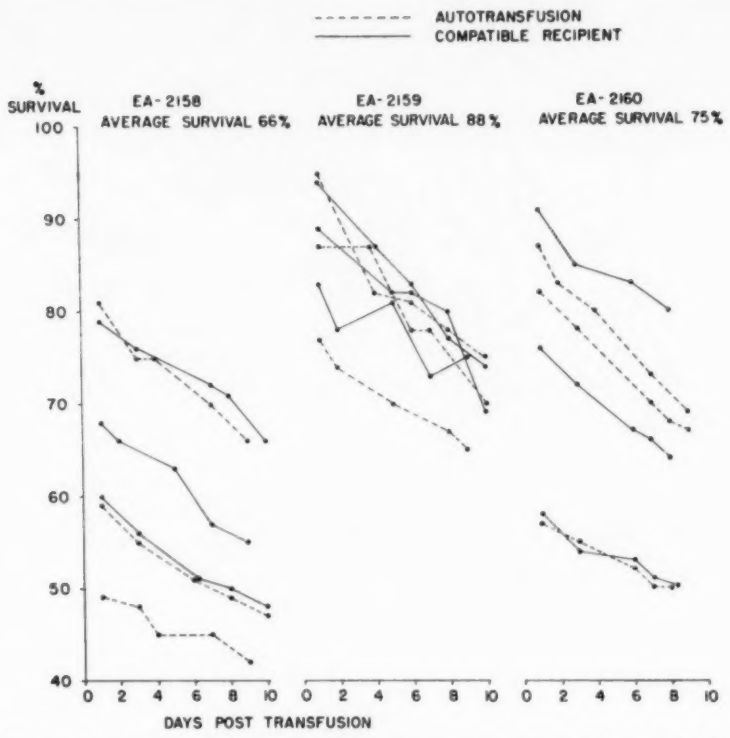

Fig. 1.—Posttransfusion Cr^{51} radioactivity survival of red cells stored as whole blood in borosilicate bottles to which various types of plastic material have been added.

Effect of the Liquid-Air Interface.—Blood from a single donor was collected in two plastic bags, one containing filtered air and one collapsed; all other factors were similar. In Experiment 1 four recipients were transfused (Fig. 2). The 24-hour posttransfusion survival of radioactivity of Cr^{51}-tagged red cells collected and stored in bags with the liquid-air interface was 68 per cent; in the control series where blood was collected without liquid-air interface, the survival was 67 per cent.

In the second experiment (Fig. 2) two recipients were used; at 24 hours posttransfusion the radioactivity survival of red cells collected and stored with the air-liquid interface was 85 per cent; identical survival was noted for the red cells collected and stored without the air-liquid interface.

TABLE II. RESULTS OF IN VITRO STUDIES ON BLOOD COLLECTED IN GLASS AND PLASTIC CONTAINERS, WITH LIQUID-AIR INTERFACE, AND SHAKEN AT DIFFERENT PERIODS OF STORAGE (EACH DATUM REPRESENTS THE AVERAGE OF DETERMINATIONS ON TWO SAMPLES)

		DAYS OF STORAGE	PLASMA HEMOGLOBIN (MG. %)	PER CENT HEMOLYSIS IN 0.63 % NaCl	ATP	DEXTROSE (MG. %)	LACTIC ACID (MG. %)	M.C.V. OF RED CELLS	pH
Shaking immediately after collection	Plastic containers	22	16	3	6.89	277	101	100	6.65
	Glass containers	21	16	5	7.08	270	102	100	6.65
Shaking after 10 days' storage	Plastic containers	22	42	1	6.22	272	130	99	6.64
	Glass containers	21	45	4	7.04	285	118	98	6.64
Shaking after 20 to 22 days' storage	Plastic containers	22	44	6	6.75	230	146	97	6.65
	Glass containers	21	71	8	6.25	247	125	98	6.70

In another experiment shaking was added to intensify the effects of the liquid-air interface in the presence of wettable and water repelling surfaces. Blood (500 ml.) was collected from young, healthy donors into a plastic bag containing 75 ml. of ACD solution. The collection was made through plastic tubing, at room temperature and with hand mixing. In each case, immediately after collection, 250 ml. of blood was transferred to a standard glass donor bottle by gravity. The plastic bag was then placed in a rigid container and partially filled with air. Thus, the liquid-air interface and the resiliency factors were practically eliminated, and the only remaining variable was the material of the container.

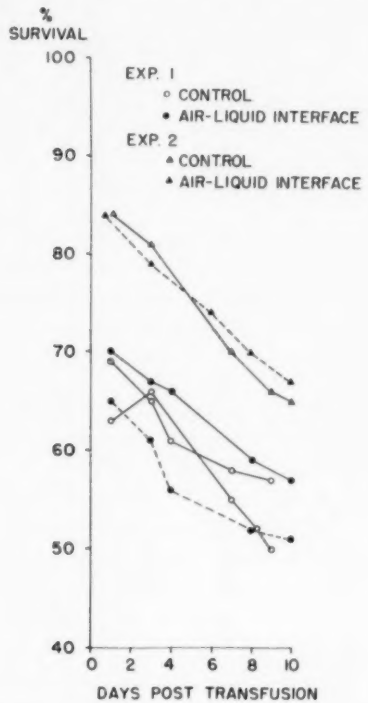

Fig. 2.—Posttransfusion Cr^{51} radioactivity survival of red cells stored as whole blood in plastic bags with air-liquid interface and in control plastic bags without the air-liquid interface.

Shaking was carried out on a mechanical agitator for about one hour, at the following intervals of time: (1) immediately after collection; (2) on the tenth day of storage; (3) after 20 to 22 days of storage, immediately before transfusion.

All blood specimens were stored for 20 to 22 days and the red cells were tagged with Cr^{51} before transfusion.

In vitro studies, immediately before transfusion, included plasma hemoglobin concentration, hemolysis of red cells in 0.63 per cent NaCl solution, A.T.P. content and mean corpuscular volume of red cells, dextrose and lactic acid concentration, and pH of plasma. No differences were found in any of the results which can be attributed to shaking or to the type of container in which the blood specimens were collected and stored for 20 to 22 days (Table II).

The results of the posttransfusion survival of red cells show that shaking of blood damages the red cells; no significant difference was found between the survival of red cells shaken in the two types of container (see Table III).

TABLE III. SURVIVAL OF Cr^{51} RADIOACTIVITY OF RED CELLS COLLECTED IN GLASS AND PLASTIC CONTAINERS, WITH LIQUID-AIR INTERFACE, SHAKEN AT DIFFERENT INTERVALS OF TIME, AND TRANSFUSED AFTER A STORAGE OF 20 TO 22 DAYS

	PLASTIC CONTAINERS		GLASS CONTAINERS	
TIME OF SHAKING	DAYS OF STORAGE	PER CENT RADIOACTIVITY 24 HR. POST-TRANSFUSION	DAYS OF STORAGE	PER CENT RADIOACTIVITY 24 HR. POST-TRANSFUSION
Immediately after collection	22	84	21	78
	22	83	21	76
	22	74	21	75
Average		80		76
At 10 days after collection	22	69	21	73
	22	71	21	69
	21	68	20	73
	21	74	20	69
Average		71		71
At 20 to 22 days after collection and immediately before transfusion	21	71	20	70
	21	67	20	70
	22	76	21	75
	22	72		
Average		72		72
Controls, not shaken, collected and stored in the same container				
	PLASTIC CONTAINERS		GLASS BOTTLES	
	21	89	21	63
	21	86	21	65
	21	84	22	93
	21	72	22	80
	21	87	20	70
	21	83	21	67
	21	93	21	53
	21	94	21	57
	21	82		
	21	82		
Average		85		69

Effect of Water Repellence and Other Physical Properties.—There has been much speculation concerning the effect of wettable and water-repellent surfaces on red cells, probably suggested by the effect noted on platelets.[4] The improved preservation of red cells stored in plastic containers has been attributed to the water repelling properties of plastic.[5] The results of the experiments previously reported on the effect of the liquid-air interface cast much doubt on the validity of this hypothesis. Agitation of blood in plastic and glass containers resulted in similar changes. More specific studies were carried out to determine the effect of wettable and water-repellent surfaces on red cells taking in consideration possible immediate effect and the effect of prolonged contact.

In the first experiment blood from 44 normal young men was collected with ACD solution as follows: in plain glass containers, 19 subjects; in silicone-coated glass containers, 14 subjects; and in plastic containers, 11 subjects. The blood specimens, about 30 ml. each, were immediately tagged with Cr^{51} and autotransfused within one hour of collection. The results of study of posttransfusion survival are shown in Table IV. No significant difference is shown attributable to the immediate effect on red cells of the 3 types of containers; the mean survival at 24 hours posttransfusion for the 3 types being similar.

TABLE IV. EFFECT OF IMMEDIATE CONTACT WITH VARIOUS TYPES OF SURFACES ON THE POSTTRANSFUSION SURVIVAL OF RADIOACTIVITY OF Cr^{51} TAGGED RED CELLS, AUTOTRANSFUSED WITHIN ONE HOUR OF COLLECTION

TYPE OF CONTAINER	PER CENT SURVIVAL OF Cr^{51} RADIOACTIVITY 24 HR. POSTTRANSFUSION	MEAN
Borosilicate glass containers	99, 97, 97, 97, 96, 95, 94, 94, 94, 94, 94, 94, 92, 90, 90, 89, 89, 89, 87	93
Silicone-coated glass containers	95, 95, 94, 94, 94, 93, 93, 92, 92, 92, 89, 89, 88, 87	92
Plastic containers	99, 96, 95, 95, 94, 94, 94, 92, 90, 89, 89	93
	Mean of 44 values = 93 Standard deviation = 3.1	

The effect of prolonged contact of red cells with different types of containers was studied in two groups in the following manner.

1. Blood was collected in plastic containers and immediately transferred to plain borosilicate bottles. In these experiments there were 3 donors and 3 transfusions, of which 2 were autotransfusions. The blood was stored from 21 to 22 days.

2. Blood was collected in glass bottles and immediately transferred to plastic bags. In this experiment 3 donors were used and the blood was autotransfused after 20 to 23 days of storage. In all instances, both donors and recipients were normal young men.

The results of posttransfusion survival studies are shown in Fig. 3. The blood collected in plastic containers and stored in glass containers shows a survival similar to the average obtained in 8 control units of normal blood collected and stored in glass containers for 21 days. The blood collected in glass containers and stored in plastic containers shows a survival similar to that obtained in 10 control units of normal blood collected and stored in plastic containers for 21 days.

Six units of blood from normal young men were collected in glass containers coated with silicone. The blood was stored in the same containers for 21 to 22 days; 4 units were autotransfused and 2 units were transfused in a compatible recipient.

The results are shown in Fig. 4. The average survival at 24 hours posttransfusion is 78 per cent, definitely higher than the average of a control group of 8 experiments using blood collected and stored in plain glass bottles for the same length of time. The survival values vary considerably in the 6 observations—from 68 to 92 per cent. The result with silicone-coated glass surfaces

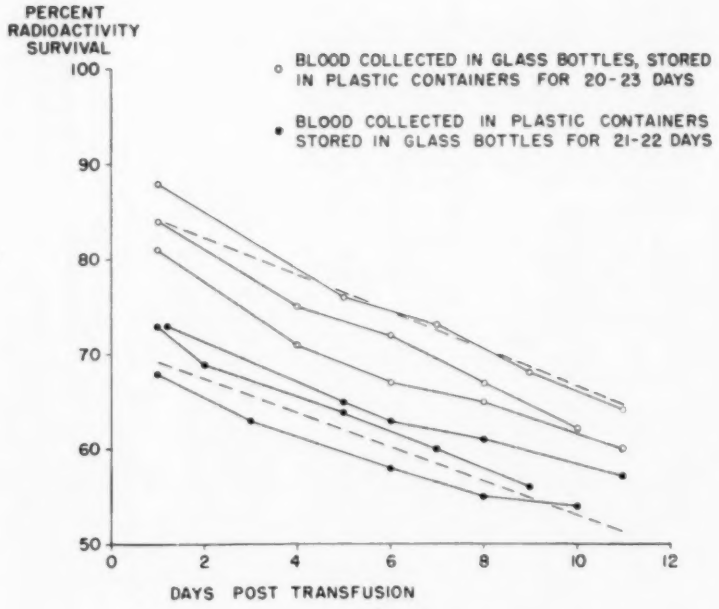

Fig. 3.

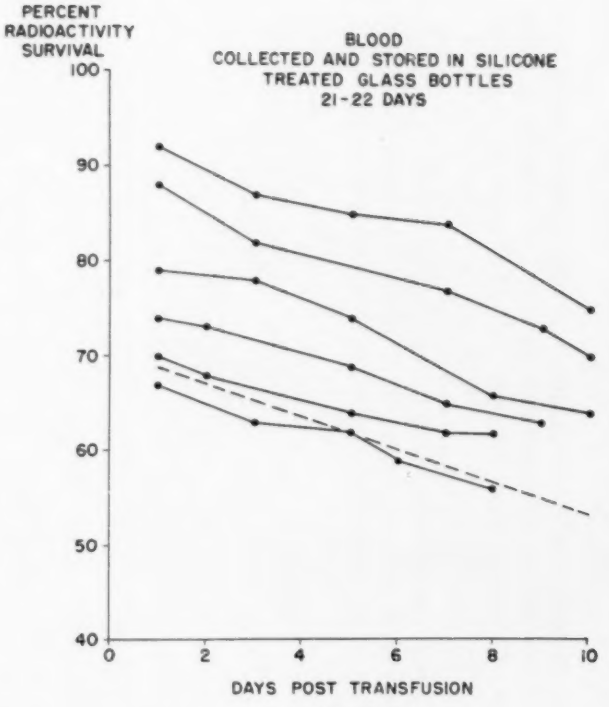

Fig. 4.

Fig. 3.—The upper interrupted line represents the average of Cr^{51} radioactivity survival of red cells in 10 transfusions of normal blood collected and stored in plastic containers for 21 days. The lower interrupted line represents the average of Cr^{51} radioactivity survival of red cells in 8 transfusions of normal blood collected and stored in glass containers for 21 days.

Fig. 4.—The interrupted line represents the average survival of red cells of 8 transfusions of normal blood collected and stored in glass containers for 21 days.

does not appear to be as good as the result obtained with the best types of plastic containers, which, at various times, have given a 24-hour posttransfusion survival of 84 to 87 per cent for red cells stored for 21 days.

DISCUSSION AND SUMMARY

Experimental evidence has been presented supporting the view that certain materials employed in the preparation of plastic containers for collection and storage of blood exert a toxic effect on the red cells. So far, polyvinyl chloride has been the only material used for the manufacture of containers for collection and storage of blood. Even high-grade polyvinyl chloride plasticized with a distilled grade of dioctyl sebacate may exert a toxic effect on red cells. This toxic effect may be eliminated when the basic plastic is compounded by the addition of a proper stabilizer, such as one containing zinc. Some plastic materials exert a toxic effect on red cells even after compounding with a stabilizer.

It has also been shown that the harmful effect of the plastic material is due to leachable substance or substances; this unfavorable effect on red cells may be detected by in vitro studies. These in vitro studies are recommended as screening tests for the evaluation of plastic materials intended for use in the manufacturing of containers for collection and storage of blood. In vivo studies, however, should always be used before releasing the containers for clinical use.

The improvement which is noted on the red cell preservation when blood is stored in glass containers treated with silicone would indicate that glass containers also release substances toxic to the red cells. When blood is collected in glass bottles containing ACD solution and the blood is removed from the bottle immediately after collection and transferred to a plastic container for storage, the properties of the ACD blood are similar to those of blood collected and stored in a plastic container. This indicates that the toxic material released by the glass is not pre-existent in the anticoagulant, but is formed from the interaction of citrated blood and container as the storage progresses. This phenomenon is probably related to the solubility of silica. At pH 5 of the ACD solution, silica is practically insoluble. At pH 6.2, the solubility of silica is 0.2 parts per million. At pH 7 of the ACD blood at time of collection, the solubility is 2 parts per million.[6] It is probable that silicates leaching out from the glass during storage of blood are responsible for the damage to red cells, reducing their posttransfusion survival. A similar conclusion applies to the effect of plastic material: the toxic effect manifests itself only on prolonged contact during storage.

It has been shown that the presence of the liquid-air interface does not influence the in vitro properties and the posttransfusion survival of red cells. No evidence has been found supporting the view that the surface properties of plastics have a causative relationship with the improved preservation of blood stored in some plastic containers. Particularly suggestive are the results of shaking of red cells in the presence of wettable and nonwettable surfaces, represented, respectively, by glass and plastic surfaces. This experiment shows

that shaking of blood immediately after collection is significantly less damaging to red cells than when done after a period of storage. This observation condemns the common practice of transporting blood after a period of storage.

The 24-hour posttransfusion survival of blood collected and stored in glass containers for 21 days has varied in experiments conducted at various times over a period of years from a mean of 69 to a mean of 74 per cent. These differences in results may be attributed to changes in the glass, probably due to reuse of the containers. Similar variations have been noted in the posttransfusion survival of blood collected and stored in glass containers coated with silicone: these variations may be due to our inability to obtain a constant and uniform coating of the glass surfaces.

CONCLUSION

The variable toxic effects noted on blood stored in glass and plastic containers is attributable to substances leached from the material of which the containers are made, either glass or plastic.

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IN VITRO STUDIES ON THE PRESERVATION OF MARROW CELLS IN GLYCEROL AT LOW TEMPERATURES

HARRY L. LOCHTE, JR., M.D.,* JOSEPH W. FERREBEE, M.D., AND E. DONNALL THOMAS, M.D.
COOPERSTOWN, N. Y.

WITH THE TECHNICAL ASSISTANCE OF LYLE NORTON, MARION WALES, AND DOROTHY THOMAS

IN LOWER animals, intravenous administration of marrow cells from hematologically normal donors will repopulate the marrow spaces and induce recovery after otherwise lethal exposure to ionizing radiation.[1-4] In man, marrow from fetal and adult cadavers and from biopsy and surgical specimens has been administered without untoward event and, at times, has seemed to be of benefit to patients receiving large doses of total-body radiation.[5]

The large quantities of normal marrow required by clinical studies may be obtained by prompt sterile dissection of hematologically normal cadavers.[6] Since suitable cadavers are not always available at the particular time when marrow is needed, some method for preserving marrow is desirable. Successful preservation allows time for testing sterility, viability, and compatibility in homologous transfers and is essential when autologous marrow is used in patients receiving extensive radiotherapy or chemotherapy.

It has been shown that mouse marrow retains its viability at 4° C. for only 3 to 5 days[7] and for only about two weeks in tissue culture.[8] Hematopoietic tissue of the mouse[9-11] and rabbit[12] have been preserved for long periods by freezing in glycerol to −79° C. In these studies on rodents, evidence of cell viability after preservation is obtained by demonstrating that intravenous administration of the preserved material prevents death after an otherwise lethal dose of radiation. In vivo assay of this type is not often practical in man, though it has been accomplished in at least one instance.[5] In vitro testing, therefore, must be used to provide preliminary information about preservation procedures applied to human marrow.

This report describes in vitro studies of the effects of glycerol and freezing on human and rabbit marrow cells. Cell counts, morphology, motility, dye permeability, and desoxyribonucleic acid (DNA) synthesis have all been used as criteria of preservation and viability. Because the synthesis of DNA is a necessary preliminary to cell division, measurement of the rate of its synthesis has particular value as an indicator of proliferative ability.[13]

From the Mary Imogene Bassett Hospital (affiliated with Columbia University), Cooperstown, N. Y.

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*Public Health Service Research Fellow, National Heart Institute, National Institutes of Health, Bethesda, Md.

MATERIALS AND METHODS

Human marrow was expressed from ribs obtained at surgery or from postmortem dissections performed within 1 to 3 hours of death. From 0.5 to 2.5 billion nucleated cells were obtained from each rib. Rabbit marrow was obtained from healthy young adult animals. The marrow was suspended in 5 per cent autologous serum or human albumin in balanced salt solution (Hanks' solution)* and was passed through stainless steel screens to break up marrow particles.[14] The suspension was centrifuged at 200 g for 10 minutes, the supernatant fat and liquid were discarded, and the sedimented cells were resuspended in serum adjusted to a pH of 7.6 or tissue culture media and glycerol-saline as indicated in the individual experiments. One to five milliliter aliquots containing 10 to 100 million nucleated cells were pipetted into 10 ml. siliconed vials for assessment of freezing procedures.

Freezing was usually carried out by placing the vials in air at −20° C. for 25 minutes. Vials were then transferred to a dry beaker floating in a Dry Ice and alcohol bath at −79° C. for 15 minutes. The frozen vials were then stored at Dry Ice temperature, −79° C. This method of "slow" freezing was found to produce a temperature curve approximating the curve described by Barnes and Loutit,[9] i.e., 1 degree per minute to −15° C., then 2 degrees per minute to −70° C. "Rapid" freezing was achieved by immersing the vials in a Dry Ice and alcohol mixture and rotating them until freezing was completed. Thawing was carried out rapidly in a 37.5° C. water bath, and glycerol was removed by a modification of the technique recommended by Sloviter.[15] This consisted of adding one-half volume of 35 per cent glucose in water to the cell suspension. One volume of saline was then added, followed by another volume of saline 5 minutes later, total dilution being 1 to 6. This diluted suspension was centrifuged at 200 g for 10 minutes and the supernatant discarded. The cells were resuspended in a 50 per cent serum and Hanks' solution and incubated for one hour in the presence of C^{14}-formate. The incorporation of labeled formate into the thymine of DNA was determined as previously described.[13] Control aliquots of marrow not exposed to glycerol or freezing were similarly incubated as soon as possible after removal from the body in order to obtain a base-line measurement of normal activity.

Cell counting was performed under the phase microscope using 5 per cent albumin in Hanks' as the diluting fluid. Motility was observed under the phase microscope. Smears were made on cover slips from suspensions of the marrow in 5 per cent albumin in saline. On a number of occasions viability was assayed by the method of Schreck[16] using eosin. Samples considered nonviable by eosin staining were nonviable by other tests. However, some samples considered viable by the eosin technique were inactive when tested for DNA synthesis.

RESULTS

The Effect of Freezing in Glycerol on Cell Counts, Motility, and Morphology.—Marrow cells exposed to glycerol and freezing have been found to be extremely fragile. Initially it was found that after removal of the glycerol the cells tended to stick together in tenacious, stringy aggregates, making cell counts and morphologic observations impossible. After a few cells are disrupted by manipulation or preservation procedures, a sticky material appears in the suspending medium. This material presumably is DNA.[17] Formation of cell aggregates in this viscous material is prevented by adding recrystallized desoxyribonuclease† to the suspending medium in a concentration of 0.01 mg. per milliliter. This purified desoxyribonuclease was found not to inhibit DNA

*Sodium chloride 8.0 Gm., potassium chloride 0.4 Gm., magnesium sulfate 0.2 Gm., calcium chloride 0.14 Gm., sodium phosphate 0.06 Gm., potassium phosphate 0.06 Gm., and glucose 2.0 Gm. Sufficient distilled water to make 1 liter; pH is adjusted to 7.6 with 0.3 N sodium hydroxide.

†Worthington Biochemical Laboratory, Freehold, N. J.

synthesis by marrow cells even in concentrations of 1 mg. per milliliter. Varidase* was found to inhibit DNA synthesis in concentrations of as little as 0.001 mg. per milliliter.

After freezing in glycerol and thawing, marrow cells were found to be easily destroyed by the usual counting procedure. Exposure to 3 per cent acetic acid resulted in cell lysis or the formation of clumps that made counting impossible. Cells incubated in vitro for one or more hours at 37° C. (Table I)

TABLE I. THE EFFECT OF THE DILUTING FLUID ON COUNTS OF HUMAN MARROW CELLS EXPOSED TO GLYCEROL AND FREEZING

DILUTING FLUID	NUCLEATED CELL COUNTS		
	INITIAL	1 HR.	4 HR.
Control			
3% acetic acid	27,750	24,100	23,800
5% albumin in Hank's solution	22,330	22,430	21,333
Glycerol and Freezing			
3% acetic acid	25,650	8,500	6,600
5% albumin in Hank's solution	21,133	20,367	20,333

are readily destroyed by ordinary counting procedures using 3 per cent acetic acid. Initially this phenomenon gave rise to the erroneous impression that cells were being destroyed by the preservation procedure. Five per cent albumin in Hanks' solution, therefore, was used as the diluting fluid, and counts were made under the phase microscope. It was then found that over 90 per cent of the nucleated marrow cells survived the glycerol and low-temperature freezing procedure.

The exaggerated mechanical fragility of preserved cells was also troublesome in making smears. Careful cover slip smears uniformly demonstrated smashing of the majority of the cells. When the smears were made using cells suspended in 5 per cent albumin in saline as described by Berenbaum,[18] it was found that the cells were morphologically normal (Fig. 1).

Marrow cells subjected to the glycerol and low-temperature procedure, and subsequently protected against mechanical trauma and clumping by desoxyribonuclease, were found under the phase microscope to be normal morphologically. They exhibited motility of approximately the same degree as the control preparations.

The Effect of "Thermal Shock" and Storage Temperature on DNA Synthesis.—In studies on the preservation of bull semen,[19] sudden chilling has been found to be lethal. Fig. 2 illustrates an experiment designed to determine if sudden chilling to 0° C. is also detrimental to marrow cells. Only slight inhibition of DNA synthesis was observed in aliquots of human marrow exposed to a single sudden chilling, but repeated chilling depressed DNA synthesis appreciably. It appears that marrow cells should not be exposed to frequent changes of temperature during in vitro manipulations.

Human marrow and rabbit marrow, suspended in 5 per cent homologous serum-Hanks' solution and stored at 4° C., maintain normal ability to synthesize

*Streptokinase-streptodornase, Lederle Laboratories, Pearl River, N. Y.

DNA for approximately 24 hours. Viability is rapidly lost at room temperature, at 37° C., and on storage at 4° C. in acid citrate dextrose solution (Formula A, U.S.P.).

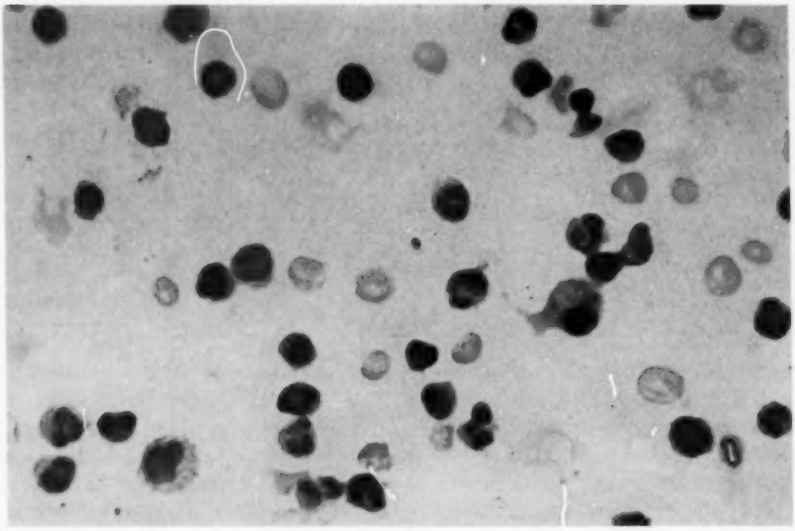

Fig. 1.—Human marrow after exposure to glycerol and freezing. Wright's stain (Magnification ×560; reduced ¼).

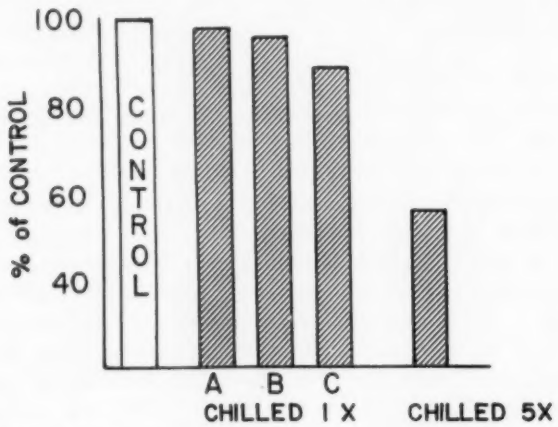

Fig. 2.—The effect of sudden chilling on DNA synthesis by human marrow cells.

The Effect of Glycerol on DNA Synthesis.—Fig. 3 illustrates an experiment designed to determine the effect of glycerol on the ability of marrow cells to synthesize DNA in vitro. It can be seen that all concentrations of glycerol in

excess of 2.5 per cent depressed DNA synthesis. This experiment indicates the necessity of removing glycerol from cells before measuring DNA synthesis.

In 4 experiments (Table II) the ability of marrow cells to synthesize DNA after exposure to 15 per cent glycerol without freezing was determined and compared with similar cells after exposure to both freezing and glycerol. It is apparent that much of the trauma of the preservation procedure is due to the exposure to glycerol.

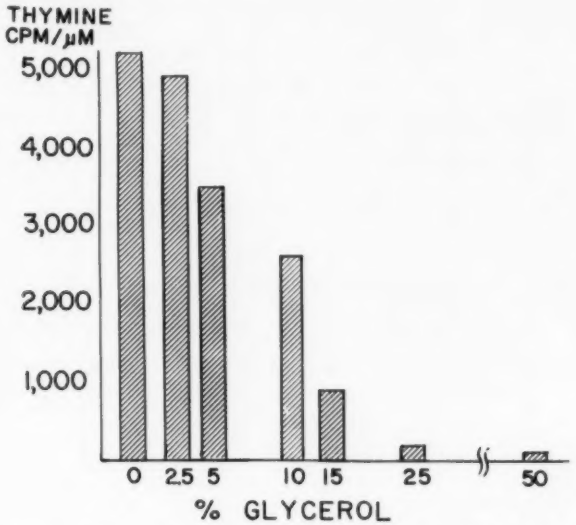

Fig. 3.—The effect of glycerol concentration on DNA synthesis by rabbit marrow.

TABLE II. A COMPARISON OF THE EFFECT OF GLYCEROL ALONE AND GLYCEROL PLUS FREEZING ON THE SYNTHESIS OF DNA BY MARROW CELLS (PER CENT OF CONTROL)

MARROW SOURCE	GLYCEROL (PER CENT)	GLYCEROL AND FREEZING (PER CENT)
Human	40	40
Human	72	53
Human	58	28
Rabbit	66	49

The Effect of Glycerol and Freezing on DNA Synthesis.—Glycerol low-temperature preservation of the ability of marrow cells to synthesize DNA is illustrated in Fig. 4, showing data from 11 experiments on human marrow and 7 experiments on rabbit marrow. A wide range of degree of preservation is evident.

Fig. 5 shows the results of 10 experiments in which preservation of ability to synthesize DNA was correlated with the percentage of erythroid cells in the marrow. A close correlation between degree of preservation and percentage of erythroid elements is evident, indicating that DNA synthesis after glycerol

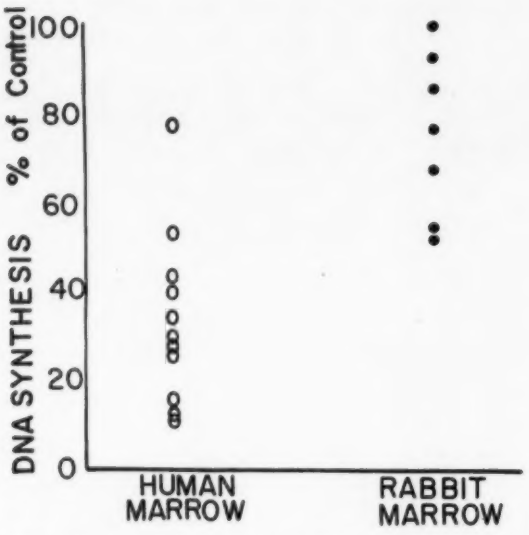

Fig. 4.—The ability of marrow cells to synthesize DNA after preservation in glycerol at -79° C.

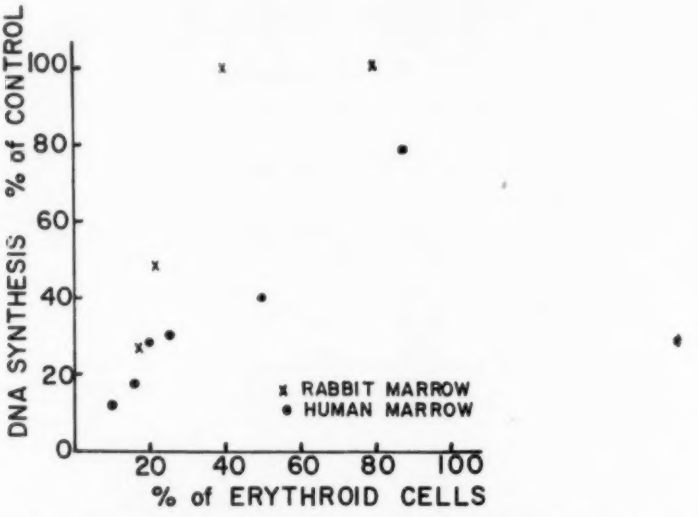

Fig. 5.—The correlation between the preservation of the ability to synthesize DNA and the percentage of erythroid elements in the marrow.

low-temperature preservation is preferentially maintained in erythroid cells. This observation may account for the variability of degree of preservation presented in Fig. 4.

In 3 experiments with preserved rabbit marrow the DNA synthesis of cells originally suspended and frozen in 50 per cent serum-glycerol was found to be twofold better than that of cells that had been suspended and frozen in 5 per cent serum-glycerol. This observation of a protective effect of high concentrations of serum during freezing in glycerol has not been confirmed with human marrow as yet.

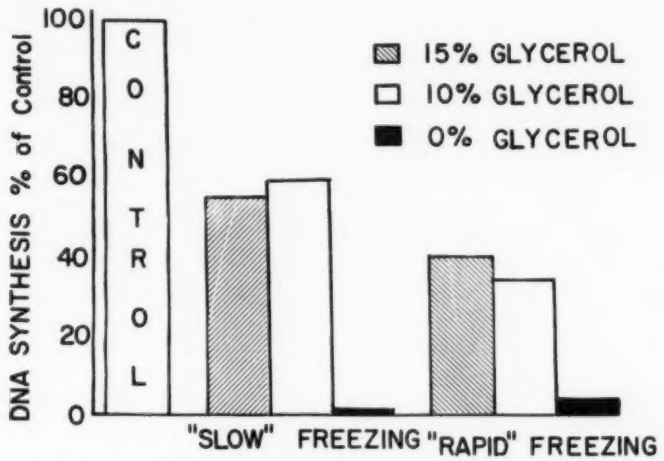

Fig. 6.—The effect of glycerol concentration and the rate of freezing on preservation of DNA synthesis by rabbit marrow.

The Effect of Freezing Rate and Glycerol Concentration on Preservation of DNA Synthesis.—Fig. 6 illustrates one of several experiments with rabbit marrow designed to determine the importance of the rate of freezing and the concentration of glycerol on the preservation of marrow cells. There is no significant difference between 10 and 15 per cent glycerol, but freezing without glycerol results in cells "nonviable" in our in vitro assays. In general, "slow" freezing permits better preservation than "rapid" freezing, but the difference is barely significant over the range studied.

The Postmortem Effect on Preservation.—Table III shows the results of 3 experiments with rabbit marrow designed to determine the effect of postmortem autolysis on marrow cell viability and marrow cell preservation. In rabbit marrow a considerable decrease in ability to synthesize DNA is apparent after 2 hours of in situ postmortem autolysis at room temperature. As indicated in Table III, rabbit marrow cells subjected to the trauma of in situ postmortem autolysis have very limited ability to withstand the further trauma of preservation with glycerol and low temperatures. Some evidence has been presented[5]

to indicate that postmortem autolytic phenomena do not seriously impair DNA synthesis in the marrow of human cadavers. The general and specific implication of these studies is that the sooner cadaver marrow is harvested, the better.

TABLE III. THE EFFECT OF TIME AFTER DEATH ON DNA SYNTHESIS BY RABBIT MARROW

EXPERIMENT	HOURS POSTMORTEM	THYMINE (C.P.M./μM)	
		CONTROL	FROZEN
A	0	7,890	9,895
	2	839	190
B	0	6,490	6,975
	3	1,021	649
C	0	7,950	2,150
	3	149	15

DISCUSSION

The studies presented show that exposure of marrow cells to glycerol and subsequent removal of glycerol accounts for an appreciable fraction of the trauma associated with the preservation procedure of glycerol at low temperatures. Trauma may occur when the glycerol enters the cell, during its presence in the cell, or during its removal from the cell. Preliminary studies of the importance of rates of addition or removal of glycerol have led to no significant improvements in the procedure. Dialysis allows slow removal of the glycerol but the progressive deterioration of marrow cells suspended in glycerol at 4° C. makes this procedure undesirable. It is possible that the removal of glycerol may be found unnecessary in the clinical use of preserved marrow. Preserved rodent marrow can be given intravenously without removal of glycerol. Preserved mouse marrow so administered is unimpaired in its ability to induce recovery after lethal irradiation.[11]

Our data indicate a relatively small difference in viability of marrow preserved by "slow" freezing, 1 or 2 degrees per minute, and the viability of marrow by "rapid" or shell freezing. Because of the small difference observed over this wide range, we have not investigated intermediate rates of freezing. For the present it appears preferable to follow the "slow" rate. However, close adherence to this rate does not seem essential.

In a previous communication[5] we reported that human cadaver marrow exhibited approximately normal rates of DNA synthesis on in vitro assay after 2 to 6 hours of in situ postmortem changes. The studies on postmortem effects in rabbit marrow reported here indicate that in situ postmortem changes cause a significant and progressive loss of ability to synthesize DNA in vitro. This loss is aggravated by subsequent application of preservation procedures which are in themselves traumatic. Hence, if useful human cadaver marrow is to be stored, it would appear to be important to make every effort to obtain collections as soon after death as possible.

The data in Fig. 5, indicating that DNA synthesis in myeloid marrow is poorly preserved by freezing in glycerol, are disturbing. If these data are substantiated, they suggest that preserved marrow may be a relatively poor source of the myeloid elements necessary for early defense against infection in the irradiated individual. It is reassuring that rabbits protected against death

from lethal irradiation by preserved marrow have shown adequate regeneration of the myeloid elements of their marrow.[12] Studies are in progress using autoradiographs with tritium-labeled thymidine to secure direct evidence about the relative effect of preservation with glycerol at low temperatures upon the preservation of myeloid and erythroid marrow cells of man.

The wide variability in the degree of preservation of human marrow and the generally better preservation of rabbit marrow deserve comment. The rabbits studied were all young healthy animals, and their marrow was obtained immediately after death. The patients from whom ribs were obtained were in the older age group and were suffering from lung disease or heart disease. The proportion of myeloid cells was greater in the human marrows than in the rabbit marrows. Obviously, young healthy individuals make the best marrow donors. The large number of marrow cells obtainable at autopsy may compensate for some potentially disadvantageous aspects of specimens obtained from older and debilitated patients.

The data presented here indicate that human marrow cells can be preserved by freezing in glycerol at –79° C. After subsequent thawing and removal of the glycerol, these marrow cells remain intact, appear morphologically normal, demonstrate motility, and exhibit an appreciable fraction of their original ability to synthesize DNA. Rodent marrow treated in comparable fashion has been kept for 6 weeks at –79° C. in glycerol and serum, and this marrow, despite impaired ability to synthesize DNA in vitro, has shown unimpaired ability to proliferate and repopulate the marrow of an irradiated host.[11] Much of the cell damage associated with the low-temperature glycerol preservation procedure is here demonstrated to be associated with the addition and the removal of glycerol. The important question is whether this damage is shared equally by all cells. The answer would appear to be that it is not. Myeloid elements suffer more than erythroid. How the essential "seed" cells make out remains to be determined. For solution of this critical problem one must turn to autoradiographs with tritiated thymidine. With the techniques described here, human marrow preserved for 8 weeks has shown no progressive deterioration. Accordingly, it seems logical to suppose that human marrow can be kept indefinitely with a useful maintenance of cells capable of proliferating in an irradiated host. The low-temperature glycerol procedure, therefore, is being continued as the basis for the current establishment of "banks" of frozen human marrow for autologous and homologous use.

SUMMARY

Recent interest in preservation of human marrow for both homologous and autologous use has indicated a need for evaluation of various preservation methods. This report describes in vitro studies of the effects of glycerol and freezing on human and rabbit marrow cells. Cell counts, morphology, motility, dye permeability, and DNA synthesis have been used as criteria of viability. When protected against mechanical and chemical trauma, and against clumping by the use of desoxyribonuclease, excellent morphologic preservation was achieved. DNA synthesis decreased after repeated chilling without freezing.

Glycerol inhibited DNA synthesis in vitro, and marrow cells exposed to 15 per cent glycerol showed moderate impairment of their ability to synthesize DNA after removal of the glycerol. The freezing to –79° C. of marrow cells in 15 per cent glycerol resulted in a further moderate decrease in ability to synthesize DNA. Myeloid cells appeared to suffer greater damage from the freezing procedure than did erythroid cells. Limited observations on the rate of freezing and the concentration of glycerol indicate that these factors are not of critical importance. An early postmortem deterioration of rabbit marrow cells was demonstrated.

The data presented here indicate that human marrow cells can be preserved by freezing in glycerol at –79° C. Preserved marrow has shown no further deterioration after 8 weeks of storage. The glycerol low-temperature method is the procedure employed in the current establishment of "banks" of frozen human marrow.

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OBSERVATIONS ON THE SITE OF PRODUCTION OF CASTLE'S INTRINSIC FACTOR IN THE RAT

F. J. Keuning, M.D., A. Arends, M.D., E. Mandema, M.D., and H. O. Nieweg, M.D.
Groningen, Netherlands

IN 1929, Castle demonstrated that the fundamental disturbance in pernicious anemia was the absence from gastric juice of a substance, which he called "intrinsic factor." The intrinsic factor is generally believed to play an essential part in the physiologic mechanism of the intestinal absorption of vitamin B_{12} (see review by Castle[1]).

A number of investigations has been performed on the site of production of the intrinsic factor, especially in the hog and in man. Recently, the rat has proved to be a suitable experimental animal for investigations of this kind. Watson and Florey[2] demonstrated that removal of the glandular part of the stomach in the rat interferes with intestinal absorption of vitamin B_{12}, as shown by increased fecal excretion of orally administered radioactive vitamin B_{12}. Extracts of the glandular part of the gastric mucosa proved to have intrinsic factor activity, because on oral administration they restored vitamin B_{12} absorption to normal values. Similar results have been reported by Nieweg and associates.[3] In these latter experiments total gastrectomy was found to result in a high excretion of orally administered Co^{60}-vitamin B_{12}. Both rat gastric juice and homogenates of total rat stomach wall or of the glandular part of the rat stomach, if administered to gastrectomized animals together with vitamin B_{12}, reduced excretion to the values found in normal animals.

In the biologic part of the present investigation the regional localization of intrinsic factor production in the rat stomach has been further studied in absorption experiments. First, the intestinal absorption of radioactive vitamin B_{12}, as measured by fecal excretion, was determined in rats in which different parts of the stomach had been separately removed. Subsequently, extracts of the different regions of the rat stomach wall were tested for intrinsic factor activity, measured as the capacity to reduce the fecal excretion of vitamin B_{12} in gastrectomized animals.

Concerning the cellular localization of Castle's intrinsic factor in the gastrointestinal tract, the evidence available so far is inconclusive. Jacobson[4] had suggested the argentaffine cells as a possible site of production. This view has been rejected by most investigators, because the regional distribution of these cells does not correspond to what is known about the occurrence of intrinsic factor. Glass and co-workers[5] isolated from human gastric juice a

From the Departments of Histology, Pathology, and Medicine, University of Groningen, Netherlands.
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strongly acidic "glandular mucoprotein" secreted, according to them, by the mucoid neck cells and containing nearly all of the intrinsic factor activity of the starting material. This acidic glandular mucoprotein, in experiments of Crevier and Bélanger,[6] was shown to consist largely of sulfomucoprotein. This result was interpreted as indicating that 3 types of gastric cells, known to produce sulfomucoproteins (Bélanger[7]), would be responsible for intrinsic factor production. These cell types are: (a) the mucoid neck cells of the fundus glands, (b) the cells of the mucous glands in the pyloric region, and (c) the cells of some few mucous glands of the cardiac-fundus transitional zone. This argumentation, however, was largely invaluated by subsequent work of Cresseri[8, 9] and particularly Gräsbeck.[10, 11]

Cresseri demonstrated that, in glandular mucoprotein preparations from hog gastric mucosa, all of the vitamin B_{12} binding capacity resided in a fraction containing neutral polysaccharide coupled to a protein moiety; it was completely absent from the acidic mucoprotein fractions. Though not conclusive, due to the nonspecific character of vitamin B_{12} binding as such, this result still suggests that intrinsic factor might be contained in the neutral polysaccharide-protein components rather than in the acidic sulfomucoproteins. Gräsbeck has more recently demonstrated that intrinsic factor activity itself in human gastric juice was definitely not associated with acidic glandular mucoprotein if denaturation was avoided by immediate cooling of the freshly collected gastric juice. In these experiments the entire intrinsic factor activity was recovered from a slow-moving zone in starch block electrophoresis; the fast-moving acidic mucoids did not show any activity.

In the histologic part of the present investigation, two approaches to the problem of cellular localization of intrinsic factor activity were tried. Sections of rat gastric mucosa were studied (1) with the periodic acid-Schiff (PAS) technique and (2) through autoradiography with $Co^{56, 57}B_{12}$.

The PAS technique is considered to demonstrate the presence of neutral polysaccharides (polyhexoses) and was chosen in view of the neutral polysaccharide-protein nature of Cresseri's vitamin B_{12} binding preparation. The aim was to investigate a possible correlation between the occurrence of PAS positive cells in the gastric mucosa and the regional localization of intrinsic factor activity as indicated by the biologic part of the present investigation.

The autoradiographic procedure, using $Co^{56, 57}B_{12}$, would seem to promise more direct information concerning the cellular localization of intrinsic factor activity through its vitamin B_{12} binding capacity, if at least nonspecific vitamin B_{12} binding could be excluded.

MATERIALS AND METHODS

Male white rats weighing 250 to 300 grams were used in these studies. The radioactive vitamin B_{12} used was labeled with $Co^{56, 57}$. Its specific activity at the time of delivery varied from 2.4 to 11.7 μc per microgram for the different batches.

For the biologic experiments the animals were operated under pentobarbital sodium (Nembutal) anesthesia. When total gastrectomy was performed, an end-to-end anastomosis between esophagus and bisected pylorus was made. Partial gastrectomies were done on the guidance of the macroscopic appearance of the gastric mucosal regions. The proximal part

of the rat stomach is covered with squamous epithelium and has a pale-gray color. In the distal glandular part, the fundus region can be easily distinguished from the antrum pylori by its more pronounced pink aspect. In the first type of partial gastrectomy both the non-glandular fore-stomach and the fundus region were resected, while the pyloric region was left intact; in the second type the fore-stomach alone was removed. These partial gastrectomies proved to be technically more difficult than total resections; leaking of the suture line with subsequent peritonitis leading to death occurred more frequently.

Extracts of the different parts of the rat stomach were made by the procedure of Watson and Florey.[2]

During the absorption experiments the animals were kept in metabolic cages and given a stock diet and water ad libitum. They were fed a test dose of 5 mμg labeled vitamin B_{12} in combination with either 0.1 ml. of saline or 0.1 ml. of an extract of stomach wall. The stools were collected over a 4-day period. After drying, the feces were weighed and ground to a fine powder. The radioactivity was measured with an end-window G.M. tube with a Philips P.W.40.31 electronic counter. The results were expressed as percentages of the test dose of labeled vitamin B_{12}.

For the histologic work the animals were killed by a blow on the head. The stomach was immediately removed and opened along the greater curvature, stretched on a cork plate, and fixed in Bouin's solution, 3 per cent formalin, or cold saturated ammonium sulfate. Afterward the material was trimmed so that the sections would contain the entire length of the lesser curvature, including small portions of the esophagus and duodenum; in addition, separate fragments of fundus mucosa were taken.

The PAS reaction was performed on paraffin sections of material fixed in either Bouin's solution or formalin; the PAS procedure of McManus[12] was used throughout. For counterstaining, hematoxylin was used.

For the autoradiographic procedure material fixed in cold saturated ammonium sulfate was used. This fixative was chosen because it has been widely used in the chemical isolation of intrinsic factor, without impairing its biologic activity. Frozen sections were incubated for 3 hours with a solution of $Co^{56, 57}B_{12}$, saturated with ammonium sulfate. The sections were subsequently washed in 3 portions of saturated ammonium sulfate, given a final short rinse of distilled water, and mounted on slides treated with gelatin-chromalum. These slides were either packed in contact with radiographic dental film (Dozahn-Schleusner) or covered with stripping film (from Kodak autoradiographic plates AR 10 and AR 50). After varying periods of exposure in the cold the material was developed.

The sections that had been in contact with dental film were stained with hematoxylin or PAS; the sections covered with stripping film were mounted unstained and viewed by phase-contrast microscopy.

In addition, a number of routine techniques were used, as indicated in the text.

RESULTS

Absorption Studies.—In a first set of experiments the fecal excretion of $Co^{56, 57}B_{12}$ was measured in rats in which either (a) the fore-stomach (cardiac region) alone or (b) the fore-stomach and the fundus region had been resected. Two groups of rats served as controls: normal animals and animals after total gastrectomy. In each experiment a single test dose of 5 mμg $Co^{56, 57}B_{12}$ was orally administered followed by 0.1 ml. of saline. The results, presented in detail in Table I, can be summarized as follows.

After removal of the *fore-stomach* alone (a), 46 per cent of the vitamin B_{12} test dose was recovered in the stools (average of 6 experiments). This

value does not differ significantly from that of the *normal* controls (12 experiments), in which 36 per cent of the vitamin B_{12} was excreted. It proves that the distal parts of the stomach produce sufficient quantities of intrinsic factor to maintain a nearly normal intestinal absorption of vitamin B_{12}.

Animals in which the *fore-stomach and the fundus region* had been resected (b) excreted an average of 70 per cent of the vitamin B_{12} test dose (6 experiments). This value compares well with the average excretion of 77 per cent in the total gastrectomy controls (11 experiments). It means that the antrum pylori, being unable to promote vitamin B_{12} absorption, fails to produce significant amounts of intrinsic factor.

These experiments together, therefore, suggest that the fundus region is either the main or the only site of intrinsic factor production.

In a second set of experiments, extracts of the fundus wall (e) and of the antrum pylori (d) were tested for intrinsic factor activity, i.e., for their capacity to restore vitamin B_{12} absorption in rats in which total gastrectomy had been performed. A control group of gastrectomized animals was treated with an extract of whole stomach wall. The results of these experiments are also recorded in Table I.

TABLE I. FECAL EXCRETION OF RADIOACTIVITY EXPRESSED AS A PERCENTAGE OF THE TEST DOSE OF $Co^{56, 57}B_{12}$ IN NORMAL RATS AND IN RATS IN WHICH DIFFERENT TYPES OF GASTRECTOMIES HAVE BEEN PERFORMED

			TOTAL GASTRECTOMY			
NORMAL ANIMALS	RESECTION OF FORE-STOMACH (a)	RESECTION OF FORE-STOMACH AND FUNDUS (b)	SALINE CONTROLS	FUNDUS EXTRACT (e)	ANTRUM PYLORI EXTRACT (d)	WHOLE STOMACH EXTRACT
27	37	55	52	44	77	36
31	37	67	62	58	82	36
32	41	68	70		88	40
32	48	68	73			42
34	54	72	74			45
34	58	92	75			46
35			79			54
36			80			57
39			86.5			58
41			89			64
43			104			
48						
Mean						
36 ± 5.6	46 ± 8.2	70 ± 11.0	77 ± 13.2	51 ± 7	82 ± 4.5	48 ± 9.3

It can be seen that fundus wall extract (e) reduced the vitamin B_{12} excretion of gastrectomized rats to 51 per cent (mean of 2 experiments). This value corresponds well to the 48 per cent found in the control group (10 experiments) treated with whole stomach wall extract and to the values found in normal animals.

In 3 experiments, in which an extract of antrum pylori was given (d), however, the average excretion of vitamin B_{12} was 82 per cent.

Previously published experiments[3] already have shown that homogenates of fore-stomach wall lacked intrinsic factor activity under similar conditions. Apparently the fundus wall is the only part of the rat stomach which contains

intrinsic factor. The two sets of absorption experiments together, therefore, clearly prove that in the rat the fundus region is the site of production of Castle's intrinsic factor.

Histologic Studies.—In the PAS-treated sections of the rat gastric mucosa, abundant PAS-positive material was present in the surface epithelium of both fundus and pyloric regions. To avoid misunderstanding it may be stated that "surface epithelium" means the lining of gastric areolae and gastric pits. The latter reach to nearly one-third of the mucosal thickness in the fundus, and to nearly two-thirds in the pyloric region.

As the fundus was the only region exhibiting intrinsic factor activity in the biologic experiments described above, its histology was of particular interest. Here, in addition to the surface epithelium, one cell type gave a positive PAS reaction: the mucoid neck cells of the fundus glands. These mucoid neck cells were found in a distinct zone approximately corresponding to the middle third of the mucosal thickness in the fundus. The lower third, occupied by the basophilic chief cells (pepsinogen cells), did not exhibit any noticeable PAS-color. Both zones did, of course, contain oxyntic cells (hydrochloric acid cells); these latter cells, as usual, were somewhat more numerous in the neck cell zone.

The cardiac region of the rat stomach is lined with nonkeratinized, stratified squamous epithelium, without any glandular structures. No PAS-reactivity was observed in this epithelium.

The pyloric region contains the usual simple tubular glands which occupy only the lower third of the mucosal thickness. These glands, although clearly of mucous character, gave only a very weak PAS-reaction.

In the adjacent duodenal mucosa only the solitary goblet cells and the submucosal Brunner's glands were PAS-positive, the latter only weakly.

These results again pointed to the mucoid neck cells of the fundus glands as possibly being responsible for intrinsic factor production. In view of the strict localization zone of these cells in the fundus mucosa, it might be expected that an equally distinct zone of blackening would be found in the autoradiographs, that is, if mucoid neck cells did contain intrinsic factor.

The contact autoradiographs of histologic sections incubated with $Co^{56, 57}$-B_{12} gave a clear picture of the regional distribution of the radioactive material. This was especially the case in longitudinal sections covering the entire lesser curvature. In Fig. 1, the (dental film) contact autoradiograph (Fig. 1, *B*) of such a section is reproduced together with its stained mother section (Fig. 1, *A*). Only one distinct region has given a definite blackening in the autoradiograph: the fundus region of the gastric mucosa. To the left the stratified squamous epithelium of the cardiac region can be seen to be entirely without radioactivity. The same holds for the pyloric region to the right. Here, only some irregular spots of blackening are present, which are partly due to some folds in the section. The duodenal part of the section, to the extreme right, also did not produce any blackening, neither in the mucosa, nor in the submucosal Brunner's glands. This regional distribution of vitamin

B_{12} binding, as demonstrated autoradiographically, completely corresponds to the regional localization of intrinsic factor activity as shown by the absorption experiments described above.

A.

B.

Fig. 1.—Longitudinal section of rat stomach along the small curvature; incubated with Co^{58}-B_{12}. *A*, Mother section stained with hematoxylin. The arrows point to transitions of cardia (C) to fundus (F), fundus to antrum pylori (AP), and antrum pylori to duodenum (D), respectively. *B*, Dental film contact autoradiograph; exposure 2½ days. Vitamin B_{12} binding is only found in the fundus region.

More detailed information concerning the localization of the vitamin B_{12} binding sites in the fundus mucosa comes from the following pictures. Fig. 2, *A*, *B*, and *C*, shows a representative piece of fundus mucosa from another experiment at higher magnification. In the hematoxylin-stained mother section (Fig. 2, *A*) the distinctly clearer zone of the mucoid neck cells can be easily seen, lying between the surface epithelium, with its configuration of areolae and gastric pits, and the darker stained zone representing the deeper portions of the fundus glands, which contain the chief (pepsinogen) cells. The (dental film) contact autoradiograph (Fig. 2, *B*) unequivocally shows the localization of vitamin B_{12} radioactivity. Quite evidently the mucoid neck cells are completely devoid of radioactivity; it is the deeper zone of the fundus mucosa, corresponding to the basophilic portions of the fundus glands, that contains all of the radioactivity. This exact correspondence to the dark-staining deeper parts of the glands is shown in Fig. 2, *C*, where the autoradiograph is seen projected over the mother section. There cannot, apparently, be much doubt concerning the localization of vitamin B_{12} binding capacity in this section. However, the poor resolution of the dental film contact method does not permit an exact appraisal of the localization on a cellular level.

Figs. 3 and 4, *A* and *B*, show the results of the stripping film autoradiography. Fig. 3 represents the transition between fundus region proper and antrum pylori in a longitudinal section. Apart from the localization in the

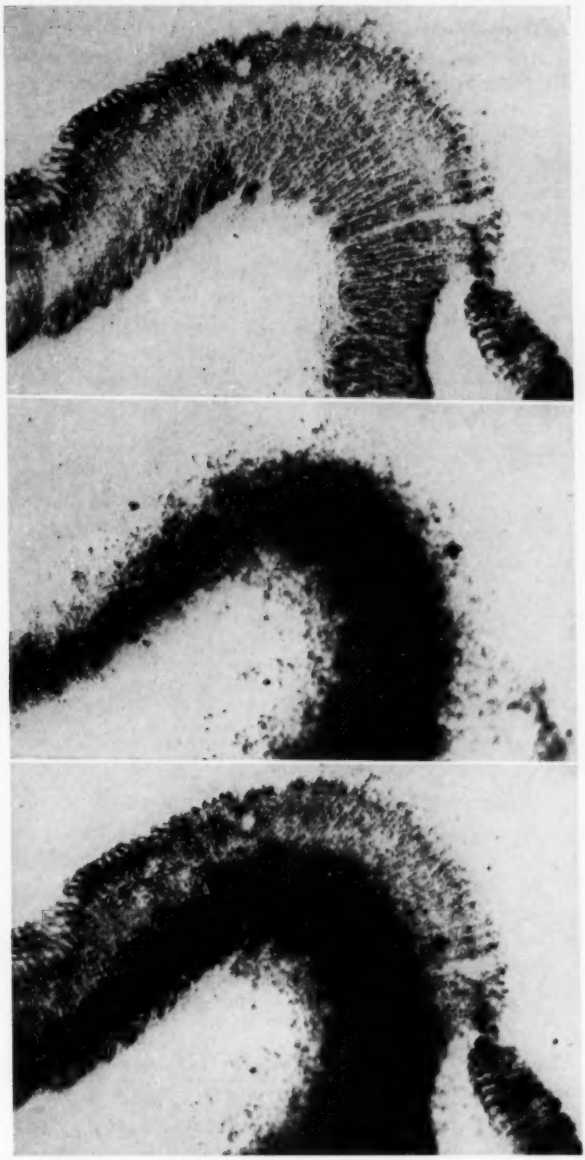


Fig. 2.—Section of rat stomach, fundus region; incubated with $Co^{56, 57}B_{12}$. *A*, Mother section stained with hematoxylin. *B*, Dental film contact autoradiograph; exposure 3 days. *C*, *B* projected over *A*. Vitamin B_{12} binding is found in deepest portions of fundus glands.

deeper portions of the fundus glands, the abrupt ending of vitamin B_{12} binding on the transition can be seen. In Fig. 4, *A*, an unstained section of the fundus with its overlying stripping film is pictured in phase-contrast. The blackening of the stripping film has the same zonal localization as in the dental film contact autoradiograph. But, in addition, it can be observed here that only the chief cells are responsible for the blackening of the stripping film emulsion. The oxyntic cells, rendered visible by phase-contrast, leave unblackened spots in the stripping film. To facilitate appraisal of the autoradiographic picture, the same is reproduced without phase-contrast in ordinary transmitted light in Fig. 4, *B*.

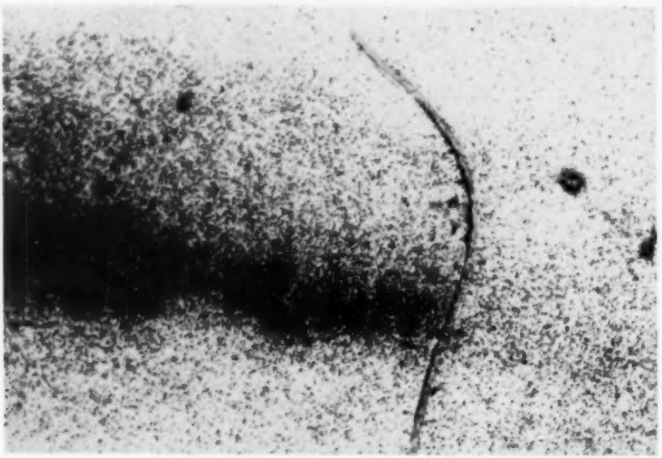


Fig. 3.—Section of rat stomach, transition of fundus region (left) to antrum pylori (right); incubated with Co^{58}-B_{12} and covered with Kodak AR 50 stripping film; exposure 16 hours. Vitamin B_{12} binding in fundus region, ending abruptly at transition to antrum pylori.

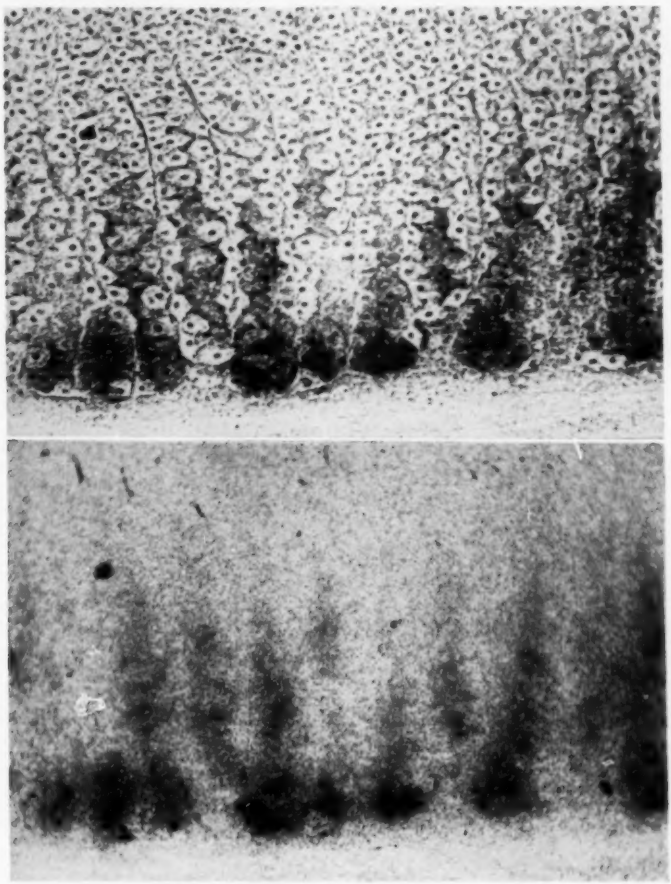
The ultimate conclusion, thus, is that under the experimental conditions used here localization of vitamin B_{12} binding and thus presumably of intrinsic factor is exclusively in the chief cells of the fundus glands. Neither the mucoid neck cells nor the oxyntic cells did exhibit any vitamin B_{12} binding. Vitamin B_{12} binding was equally absent from the mucous surface epithelium of areolae and gastric pits in fundus and pyloric regions, and in the mucous glands of the pyloric region. The cardiac region and duodenum also were entirely devoid of vitamin B_{12} binding capacity.

Control Experiments.—The rather unexpected outcome of the autoradiographs induced us to perform several control experiments.

First, a number of paraffin sections from gastric and duodenal wall material fixed in Bouin's solution were stained with the two Bodian-Protargol methods modified according to Dawson[13] for argentaffine cells. Clear pictures were obtained showing these cells scattered singly along the entire length of the gastric glands, both in the fundus and pyloric regions. They were present

in even greater numbers in the duodenal mucosa. Neither the regional distribution nor the scattered distribution in the mucosa did show any correspondence whatsoever with the vitamin B_{12} localization observed in the autoradiographs.

A.



B.

Fig. 4.—Section of rat stomach, fundus region; incubated with $Co^{56,57}B_{12}$ and covered with Kodak AR 10 stripping film; exposure 24 days. *A*, Phase-contrast photomicrograph. *B*, Photomicrograph in transmitted light. Vitamin B_{12} binding in chief (pepsinogen) cells of fundus glands.

Second, a number of experiments were performed to ascertain the presence of biologically active intrinsic factor in the sectioned material at the moment of incubation with vitamin B_{12}. Fundus wall was fixed in cold, saturated ammonium sulfate, homogenized in a Bühler blender at 4° C. and the homogenate

dialyzed against cold saline. After high-speed centrifugation, the supernatant was tested in totally gastrectomized rats, as described above, and proved to have intrinsic factor activity only slightly less or equal to that of native gastric wall homogenates.

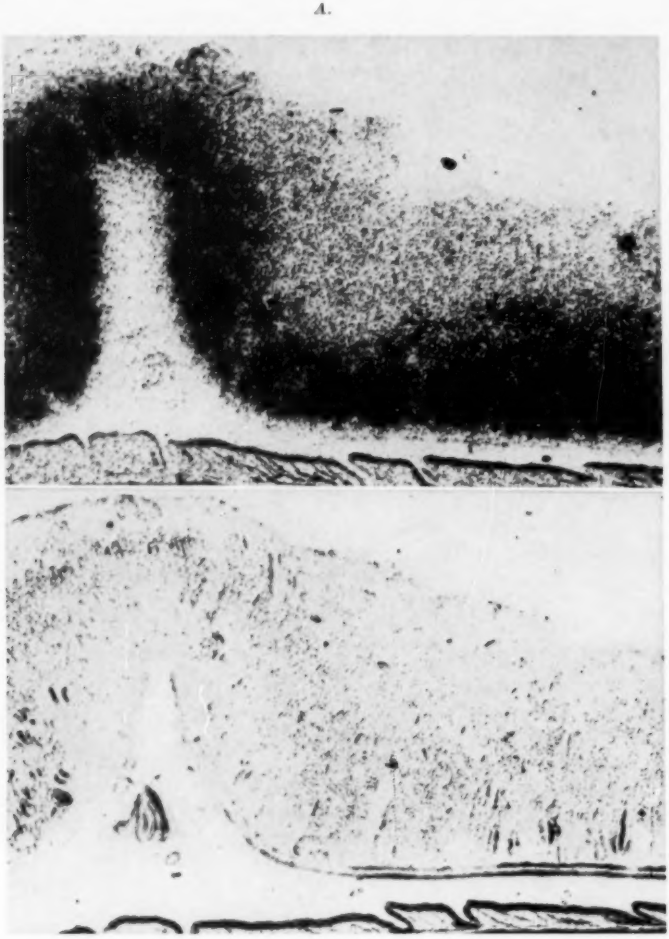


Fig. 5.—Consecutive sections of rat stomach, fundus region. *A*, Incubated with Co^{56}, $^{57}B_{12}$ (control section). *B*, Heated to 100° C. for 3 hours and incubated with Co^{56}, $^{57}B_{12}$. Both sections covered with Kodak AR 50 stripping film; exposure 24 hours. Vitamin B_{12} binding destroyed by heating.

Third, an experiment was undertaken in which rat pancreatic material was given the complete autoradiographic procedure parallel to the gastric material from the same animal. No vitamin B_{12} binding was seen in pancreatic

cells. This result excludes the possibility that nucleoprotein material, which is present in comparable quantity and distribution in the chief cells of the fundus glands and in the exocrine pancreatic cells, as such might be responsible for vitamin B_{12} binding in the fundus chief cells.

Last, control sections of the fundus region were heated to 100° C. in saturated ammonium sulfate for 3 hours, and otherwise treated parallel to the experimental sections. Fig. 5, *A* and *B*, representing autoradiographs from two consecutive sections of fundus mucosa, clearly shows that heating completely destroyed the vitamin B_{12} binding capacity. The resulting autoradiograph (Fig. 5, *B*) is entirely negative, whereas the control section presents the usual autographic pattern.

DISCUSSION

The biologic part of the present investigation, i.e., the experiments with partial gastrectomies and the substitution experiments with stomach wall extracts, has shown beyond any reasonable doubt that in the rat the fundus region of the stomach is the site of production of Castle's intrinsic factor. The substitution experiments with the extracts of fundus wall and pyloric regions, if taken by themselves, may not seem entirely convincing in view of their small numbers. All data taken together, however, show a striking mutual confirmation which clearly increases their significance.

The autoradiography presented a well-defined picture of the localization of vitamin B_{12} binding in the rat gastric mucosa. Regarding the regional localization of intrinsic factor, the autoradiographs clearly support the biologic experiments: a selective vitamin B_{12} binding was found in the fundus region. At a cellular level the localization of vitamin B_{12} binding was unequivocally in the chief (pepsinogen) cells of the fundus glands.

The autoradiographs gave no indication whatsoever that the mucoid neck cells play a part in the production of the intrinsic factor. Our results clearly suggest that the chief cells are responsible for the elaboration of the intrinsic factor in the rat.

This interpretation, however, needs a critical analysis. First it should be envisaged that the observed cellular localization might represent a diffusion artefact, as is often seen in histochemistry. This possibility can safely be ruled out because intrinsic factor is precipitated by saturated ammonium sulfate, the fixative used. Second, it is obvious that the autoradiographic technique used will only demonstrate vitamin B_{12} binding and not intrinsic factor activity itself. This is an essential point, because quite a number of unrelated substances have some degree of vitamin B_{12} binding capacity, although most authors agree that intrinsic factor is the most active among them. There is, however, good evidence that, under the conditions of our experiments, vitamin B_{12} binding represents the presence of intrinsic factor. A first argument is that our procedure, with saturated ammonium sulfate as a fixative, was shown not to destroy intrinsic factor activity in the pertinent control experiment. This means that, for all we know, intrinsic factor must have been responsible for most, if not all, of the vitamin B_{12} binding observed. A second argument

comes from a control experiment; upon heating to 100° C., a temperature known to destroy intrinsic factor, the sections completely failed to take up vitamin B_{12}.

We believe, therefore, that our autoradiographs are fairly conclusive with regard to the localization of intrinsic factor in the chief cells of the fundus glands, and, consequently, that these cells should be considered as the presumable site of production of intrinsic factor in the rat.

Cytologically, the chief cells of the fundus gland have the typical characteristics of protein-synthesizing cells, whereas any sign suggesting the production of a mucoid or polysaccharide is lacking. This suggests that the active principle of the intrinsic factor might be a protein or a polypeptide rather than a mucoid or a polysaccharide. This hypothesis is in agreement with the observation of Cresseri,[8] cited above, that the vitamin B_{12} binding substance in electropherograms of glandular mucoprotein (which presumably contains intrinsic factor) contained a protein moiety as shown by its bromphenol staining.

However, it should be mentioned that experiments with hog gastric mucosa, now in progress, have shown that conditions in this animal are more complicated than in the rat. This holds for the biologic and perhaps also for the chemical properties of intrinsic factor as well as for the presence of substances exhibiting nonspecific vitamin B_{12} binding. Species differences like these might be responsible for much of the confusion in the intrinsic factor problem.

SUMMARY

Two series of experiments were performed with the purpose of determining the site of production of Castle's intrinsic factor in the rat.

The intestinal absorption of radioactive vitamin B_{12} was studied in rats in which different parts of the stomach had been resected. The data obtained in these animals indicated that the fundus is essential for normal vitamin B_{12} absorption and, therefore, can be considered responsible for the production of the intrinsic factor. This was confirmed by testing extracts of the different parts of the rat stomach for intrinsic factor activity in rats in which total gastrectomy had been performed.

In the second part of this study a number of histologic techniques were applied in an attempt to determine the type of cell responsible for the secretion of the intrinsic factor. The most significant information was obtained by autoradiography of sections incubated with radioactive vitamin B_{12}. Selective uptake of vitamin B_{12} occurred in the chief (pepsinogen) cells of the fundus glands. Presumably this cell type elaborates Castle's intrinsic factor in the rat.

We wish to express our sincerest thanks to N. V. Philips-Roxane, Weesp-Holland, for the generous gifts of radioactive vitamin B_{12}. Their willingness to take up the preparation of this valuable and costly material provided us with the main tool for the present investigation.

The histologic work, including autoradiography, was done by Miss E. Binkhorst (Department of Histology). Microphotography was by Dr. J. Wachters (University Photographic Department). Dr. J. Abels assisted in some of the biologic control experiments.

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EVALUATION OF A METHOD OF DIRECT LEFT HEART PUNCTURE

MARIA Z. GRAJO, M.D.,* AND PAUL R. LURIE, M.D.
INDIANAPOLIS, IND.

PERCUTANEOUS needle puncture of the left ventricle has been advocated as a relatively safe method for pressure measurement and cardioangiographic study. It has been done for the most part in adults, and is performed only occasionally in children.[1-6] While it is often easy to pass a venous catheter into the left side of the heart of infants through the probe-patent foramen ovale, when access to the left side is most urgently needed it may be impossible to gain. It seemed desirable to develop a method which would have special attributes for work with infants and small children. These would be: (1) a simple aiming technique which would assure puncture of the intended chamber despite absolute and relative variation in size of structures from patient to patient; (2) minimal effect of practice upon the likelihood of success in the event that the procedure was utilized only occasionally; and (3) use of a tube of sufficiently large bore to permit satisfactory contrast injections for cardioangiography. It is believed that the method to be described below promises to fulfill these criteria in patients. It has the added advantage of permitting catheterization from the ventricle through the aortic valve and retrograde through the mitral valve. This paper reports the trial of the method in a series of 12 rabbits. This experimental animal was selected because of the smallness of the heart, thus pitting the method against a maximal handicap. It was also tried in 6 mongrel dogs to study its use in larger hearts. Finally, left ventricular puncture using this approach was performed successfully and apparently harmlessly in a 3-month-old infant with severe congenital heart disease.

MATERIALS AND METHODS

A stylet (Fig. 1, *A*) made of a 19 gauge steel piano wire, 16 cm. in length was sharpened at one end to a needle point, and bent at a right angle 1.5 cm. from the other end. A piece of polyethylene† or tetrafluorethylene‡ tubing (Fig. 1, *G*) through which the wire could be threaded snugly was cut. A piece 1.5 cm. long (Fig. 1, *D*) was cut from a 16 gauge hollow needle§ and inserted through one end of the tubing. This necessitated slight stretching of the plastic, the internal diameter of which approximately equaled that

From the Department of Pediatrics, Indiana University School of Medicine, Indianapolis, Ind.

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*Dr. Grajo is a Research Fellow of the East Central Indiana Heart Foundation.

†Intramedic: internal diameter, 0.047 inch; outside diameter, 0.067 inch. Clay-Adams, Inc., New York, N. Y.

‡Teflon (Dupont) thin-walled spaghetti tubing No. SP-600, AWG 18; internal diameter, 0.042 inch; outside diameter, 0.066 inch. Sparta Manufacturing Company, Dover, Ohio.

§Standard needle, Becton, Dickinson & Company, Rutherford, N. J.

of the 16 gauge needle. To this segment of needle, a removable adapter* (Fig. 1, *B*, *C*, *E*, and *F*) was fitted. The stylet was threaded through the adapter and tubing so that only its sharpened end protruded. A piece of No. 7 cardiac catheter (Fig. 1, *H*) 3 mm. in length was then passed over the tubing to serve as a marker.

For use in infants and small children, the stylet was replaced with a 19 gauge hypodermic needle, 25 cm. in length and joined to the hub of a 16 gauge needle. A tetrafluorethylene tubing was used, the end of which was cut and shaped to fit the point of the needle.

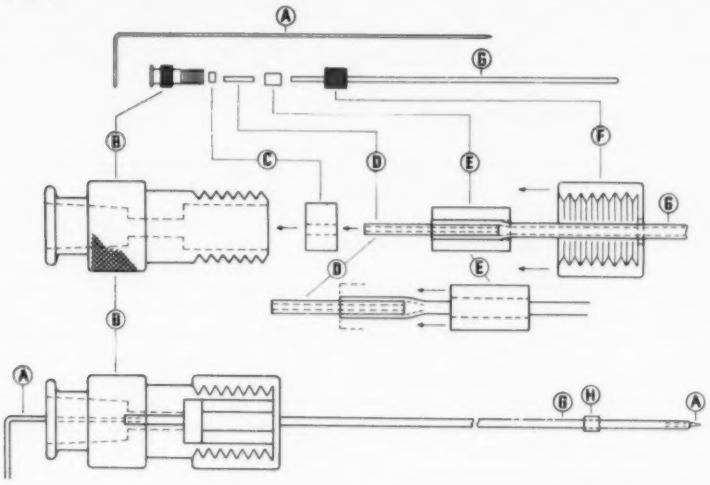

Fig. 1.—Stylet tubing instrument for percutaneous ventricular puncture. *A* = stylet; *B* = adapter body; *C* = rubber gasket; *D* = piece of 16 gauge needle; *E* = steel gland; *F* = steel gland nut; *G* = polyethylene or Teflon tubing; *H* = depth marker.

1. *Animal Experiments.*—Twelve rabbits with an average weight of 3 Kg. were obtained.

An unanesthetized rabbit was strapped by its legs to a board. The anterior chest was shaved and cleansed with Zephiran solution 1:1,000. ECG needle electrodes were placed subcutaneously in the legs. A skin stab wound 2 mm. in length was made over the left paraxiphoid area. The stylet and polyethylene tubing (Fig. 1, *A* and *G*) were inserted through the incision and subcutaneous tissues, and into the thoracic cavity. Under fluoroscopic control with an image intensifier, the tip was directed toward the apex of the heart at a 35 degree angle and very slightly to the right. As soon as pulsations were felt, the stylet tubing was quickly thrust forward into the heart, the marker (Fig. 1, *H*) being placed at an estimated proper distance of insertion so as to serve as a safety guide. When the ventricular cavity was entered, the stylet was removed and the tubing was advanced slightly. A 3-way stopcock was attached to the adapter (Fig. 1, *B*). Ventricular pressures were recorded with a Hathaway impedance gauge. The tubing could be advanced and retracted easily in various directions. It was filled with contrast medium† which rendered it visible; then the 3-way stopcock was rotated to connect the tubing with the pressure gauge, without losing the contrast medium. This permitted the tube to be seen and the pressure in the chamber to be constantly observed on the oscilloscope. The tubing was passed into the outflow artery, and into the atrium. It was occasionally flushed with 1 to

*U. S. Catheter & Instrument Company, Glens Falls, N. Y.
†Hypaque M 90 per cent, Winthrop Laboratories, New York, N. Y.

2 ml. of normal saline solution, containing 1 mg. of heparin in 500 ml. of fluid. After pressure measurements were taken, the tubing was removed. Light pressure and adhesive tape were applied over the wound. The rabbit was freed and returned to its cage.

Gross postmortem examination was performed in rabbits that died shortly after the procedure. Histologic studies were done in those which were killed with pentobarbital sodium given intravenously 8 or 9 days, 16 days, 36 days, and 43 days after the ventricular puncture.

The procedure was tried in 5 anesthetized mongrel dogs, weighing 11.5 to 13.5 kilograms. The stylet and tubing were inserted through the fourth left intercostal space anteriorly. Four of these were used in other experimental studies, and were sacrificed after being subjected to ventricular puncture. One was kept alive and sacrificed after 3 weeks.

The heart was exposed in an anesthetized dog weighing 24 kilograms, the pericardium being left intact. The stylet and tubing were thrust successively through the anterior wall of the left and right ventricles, then into the left and right atria. The pericardium was opened, and the 4 chambers were again punctured. The main left anterior descending coronary artery and 2 of its terminal branches were also punctured with the stylet.

2. *Clinical Trial in One Case.*—S. P., a 3-month-old infant weighing 11 pounds and 7 ounces, had repeated respiratory tract infection and pulmonary congestion, cardiomegaly, and enlarged liver. Right heart catheterization and selective cineangiocardiography were inadequate in arriving at a diagnosis because of inability to localize the shunt by blood oxygen saturation studies, and poor visualization of the left side of the heart and aorta due to insufficient concentration of contrast medium. The possible diagnoses entertained were truncus arteriosus; common atrioventricular canal; and ventricular septal defect, alone or combined with an atrial septal defect, patent ductus arteriosus, or coarctation of the aorta. Left heart studies were done to arrive at a more definitive diagnosis. The patient was anesthetized with intramuscular meperidine hydrochloride and secobarbital sodium. The fifth left intercostal space at the midclavicular line was infiltrated with procaine hydrochloride; a stab wound of the skin 2 mm. long was made. The instrument was thrust in through the apex of the heart. A 1 ml. syringe containing 70 per cent Urokon was attached to the stylet needle. When the point was shown to be in the ventricle by aspiration of blood into the syringe, contrast medium was injected as the needle was retracted and the tubing slightly advanced.

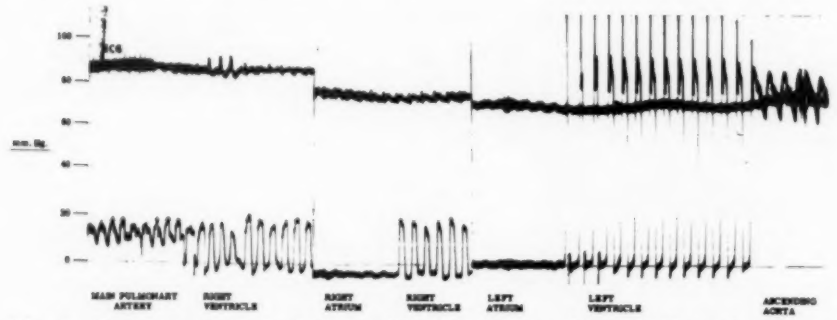

Fig. 2.—Pressure tracings in both ventricles through the atrioventricular and semilunar valves in rabbits. Note systolic overshoot artefact in left ventricle.

RESULTS

The rabbit's heart was not fixed in the mediastinum. It was easily displaced by just placing the stylet tubing in the thoracic cavity and feeling for

ventricular contractions. Once the ventricular cavity was entered, the pulsations imparted by the heart to the stylet diminished or disappeared. If the tubing was in the left ventricle, bright red blood spurted from it. Dark blood flowed slowly from the tubing if the right ventricle had been entered instead.

TABLE I. PHYSIOLOGIC DATA*

RABBIT NO.	COURSE OF TUBING	PRESSURES	ECG
1	LV-AA-LV	AA = 63/49 LV = 67/–6	CR = 240/min. (during puncture) CR = 120/min. (end of procedure)
2	LV-AA	LV = 66/–1 AA = 65/51	CR = 180/min.
3	RV-MPA-RV	RV = 16/–5 MPA = 18/4	CR = 260/min. PVC, 1 to 3, with tubing in RVO
4	LV-AA-LV-LA-LV	AA = 106/85 LV = 104/–2 LA = 0.5 mm.	CR = 240/min. Slight S-T elevation with tubing in LV; none in AA or LA
5	RV-MPA-RV	MPA = 8/4 RV = 10/–6	CR = 200/min. Rare PVC with tubing in RVO
6	RV-RA-RV	RV = 17/–6 RA = –9 mm.	CR = 240/min.
7	LV-AA-LV-LA-LV	LV = 97/–0.8 AA = 95/82 LA = 1.3 mm.	CR = 320/min.
8	LV-LA-LV-AA	AA = 116/96 LV = 112/–2 LA = –6.7 mm. LV = 109/–2	CR = 200/min.
9	LV-AA-LV-LA	AA = 94/85 LV = 103/0.6 LA = –6.4 mm. LV = 107/–5	CR = 300/min. S-T elevation
10	RV-RA	RV = 15/–2 RA = –6 mm.	CR = 220/min.
11	LV-AA-LV-LA	LV = 86/–1 AA = 89/70 LA = –2 mm.	CR = 200/min.
12	RV-IV septum-LV-AA-LV-LA-LV-RV-RA-RV	LA = –7 mm. LV = 84/–0.3 AA = 79/63 RA = –7 mm. RV = 14/–2	CR = 260/min. S-T elevation Few PVC with tubing in RVO

*Key to Abbreviations: LV = left ventricle; AA = ascending aorta; LA = left atrium; RV = right ventricle; MPA = main pulmonary artery; RA = right atrium; RVO = right ventricular outflow; IV = interventricular; CR = cardiac rate; PVC = premature ventricular contractions.

The tubing was passed into the main pulmonary artery or aorta, and into the left or right atrium, depending on which ventricle was entered. Good pressure curves were obtained (Fig. 2 and Table I). In Rabbit 12, the stylet pierced the right ventricle and went through the interventricular septum into the left ventricle. Pressure tracings were obtained from both ventricles and atria and the ascending aorta. Incidence and amount of bleeding were increased considerably when more than one attempted puncture was made, and bleeding was

the cause of death in 3 animals. A fourth animal was sacrificed 30 minutes after the end of the procedure, as 4 attempts had been made. Gross examination in this case did not show enough bleeding to have presumably resulted in death.

TABLE II. PATHOLOGIC FINDINGS

RABBIT NO.	SITE AND NO. OF PUNCTURES	TIME OF DEATH	AUTOPSY*	
			BLEEDING	HISTOLOGY
1	LV (4)	Sacrificed after 7 days	+	–
2	LV (3)	Sacrificed after 8 days	0	+++
3	RV (1)	Sacrificed after 36 days	0	++++
4	LV (2)	Died after 20 min. of hemorrhage from puncture wound in ascending aorta	++++	–
5	RV (1)	Sacrificed after 16 days	0	No lesion
6	RV (2)	Died after 40 min. of hemorrhage from puncture wounds in RV	++	–
7	LV (4)	Sacrificed at end of procedure	+	–
8	LV (1)	Sacrificed after 43 days	0	++
9	LV (1)	Sacrificed after 16 days	+	+++
10	RV (2)	Sacrificed after 9 days	+	++
11	LV (1)	Sacrificed after 9 days	++	++
12	RV (4)	Died after 5 to 12 hr. of hemorrhage from puncture wound	+++	–

*Bleeding: 0 = clear pericardial cavity; + = minimal pericardial sanguinous fluid; ++ = small blood clot in pericardial cavity or in mediastinum; +++ = unilateral hemothorax and bloody pericardial fluid; ++++ = bilateral massive hemothorax.

Histology: – = no examination; + = necrosis of tissues, focal hemorrhage, polymorphonuclear infiltration; ++ = ingrowth of blood vessels, fibroblasts, macrophages; +++ = lymphocytes, plasma cells, prominent collagen; ++++ = contracted scar, avascular.

Other observations made were lowering of the cardiac rate when 4 thrusts were made (Rabbit 1 in Table II), transient ventricular extrasystoles on touching the ventricular wall externally in most cases, and occasional ST-T changes. Pneumothorax was looked for in each case, and was not observed fluoroscopically.

In Table II, the amount of bleeding and the extent of fibrosis are shown in relation to the site and number of puncture wounds and the age of the lesions. Macroscopic examination showed that a coronary artery was hit twice in the middle of its course during the experiment in the first animal; and slight sanguinous fluid was noted in the pericardial cavity when the animal was killed after 7 days. Histologic abnormalities were confined to the path of the instrument. As early as 8 days, scar formation was evident. In all instances, the pericardium and endocardium were intact with no thrombus formation.

In dogs, except for some difficulty in going through the chest wall, the procedure was easier than in rabbits. The Teflon tubing remained firmly extended over the stylet despite the resistance offered by the tissues in contrast to the thin-walled polyethylene tubing which tended to fold in "accordion" fashion. When the thoracic cavity was reached, the left ventricle was punctured with accuracy at one trial. Not only was the target bigger, but the left ventricle was thicker, so that bleeding into the pericardial cavity did not occur even after withdrawal of the tubing. Gross examination showed clear pericardial cavities; in one, there was a small subpericardial hemorrhage at the site of puncture.

When multiple punctures were done through the different chambers and coronary vessels in the dog's exposed heart, slight bleeding occurred when the pericardial vessels were pierced. There was no bleeding from the wounds in the ventricular wall. Blood oozed from the atrial puncture wounds in negligible amounts when the pericardium was intact. With the pericardium open, the second atrial punctures did not cease bleeding until direct pressure was applied. Blood spurted when the left anterior descending coronary artery was pierced, and oozed from the corresponding vein when the latter was punctured in turn. There was minimal bleeding when the terminal coronary artery branches were subjected to puncture.

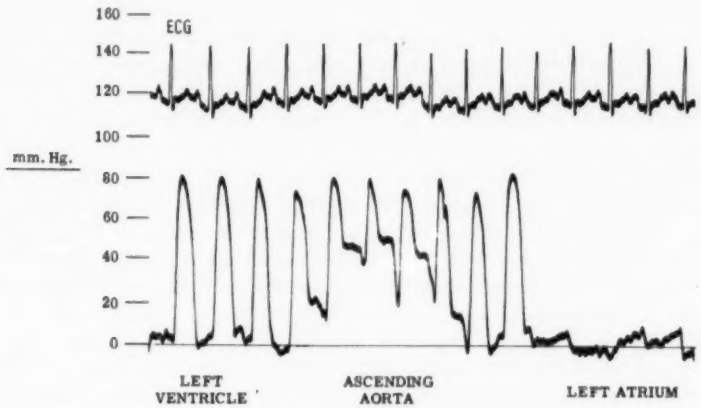

Fig. 3.—Pressure recording taken during manipulation of catheter through the precordium of a 3-month-old infant. The low aortic diastolic pressure in some of the complexes indicates aortic insufficiency. The variability of this finding suggests that it is an artefact due to pressure of the catheter on the valve.

In the 3-month-old infant, moderate resistance was offered by the subcutaneous tissues and the hypertrophied left ventricular wall. The left ventricle was entered without undue arrhythmia. The tubing was passed into the aorta and left atrium, and pressure recordings were taken (Fig. 3). However, the tip of the tubing slipped back into the pericardial sac before satisfactory injection of contrast medium could be made. A second puncture was made with greater ease, and left ventricular cineangiocardiography was performed. The study showed a ventricular septal defect and narrowing of the aorta, but no discrete coaretation. The child tolerated the procedure well, and was wide awake and fairly active when brought back to the ward. Follow-up over a 1-month period indicated no evidence of delayed ill effects.

DISCUSSION

It is believed that this new method has distinct advantages over needle punctures into the left atrium or left ventricle for measurement of pressures or injection of contrast medium directly, and over the polyethylene tubing

threaded through a needle and passed subsequently through the different cardiac chambers. Ordinarily, 15 to 18 gauge needles are used for left heart punctures through the chest wall, and one corresponding to a 22 gauge needle by the suprasternal approach. The stylet or needle used in our studies is of 19 gauge. Since the stylet is threaded through the plastic tubing, and removed after it is in a ventricular chamber, a large bore tubing remains in place. This tubing corresponds in outside diameter to a No. 5 French cardiac catheter. It is about twice the external diameter of the left heart catheterization tubing passed through a 17 gauge transbronchial needle. Its larger lumen lessens the inciden e of clotting, and enables one to get better pressure tracings and inject a satisfactory amount of contrast medium rapidly. The ventricular wall can clamp more effectively on the slightly compressible tubing than on a needle, preventing leakage. Being flexible, this tubing produces less fixation of the heart and should be better tolerated than a rigid needle of the same or larger size. Furthermore, the tubing is of sufficient size and rigidity to permit considerable manipulation in achieving passage through the atrioventricular and semilunar valves. Reasonably accurate pressure curves are obtainable through both the polyethylene and Teflon tubes.

The rabbits used for the study were young, and had hearts which weighed about 4 grams, as compared to about 100 grams in dogs weighing 12 kilograms. Considering the size of the hearts and the thickness of the ventricular wall in relation to the size of the puncture wound in the myocardium, there was remarkably little bleeding into the pericardial and thoracic cavities when the first attempt was successful. Possibly a pericardial or internal mammary vessel was punctured in the eleventh animal to account for the blood clot in the inferior mediastinum. In the first rabbit, there was gross evidence that a major coronary artery had been pierced twice. There were only minimal S-T changes observed while the experiment was in progress. Unfortunately, an electrocardiogram was not taken before the rabbit was sacrificed to look for further changes, if any. Presumably, the rabbits had normal myocardia so that the rate of healing was fairly rapid.

In dogs, except for some difficulty in going through the chest wall, the procedure was easier than in rabbits. The diaphragm of the dog is more conical and higher. It was difficult to avoid the left leaf of the diaphragm by the paraxiphoid approach. The stylet and tubing, therefore, were inserted through the fourth left intercostal space. Apparently the wounds produced in the left ventricular wall closed immediately upon withdrawal of the tubing so that bleeding was not observed to come from them. Autopsy studies have been reported on dogs punctured with a 17 gauge needle; the sites of entry were often difficult to identify, and detected only microscopically as focal scarring.[4]

As shown by multiple thrusts into the exposed heart of a dog, it could be that even in our rabbit experiments, bleeding did not always come from the puncture sites of both ventricles, but possibly came from the pericardial vessels or coronary blood supply when these were pierced by the stylet.

For infants and small children, a piece of tetrafluorethylene tubing, 25 cm. in length was thought sufficient to reach from the apex of the heart to the

proximal aorta. A 19 gauge hypodermic needle was used instead of a wire stylet in order to indicate whether the tip of the instrument was inside the pericardial cavity or in a cardiac chamber before removal of the needle, and also, in order to avoid air embolism, by permitting the introduction of contrast fluid into the tube as the needle was withdrawn. Approaching the left ventricle through the apex reduced the possibility of piercing large branches of the coronary arteries. Also, the internal mammary vessels were avoided. However, the pleural space was probably traversed. The procedure should be tolerated by the ventricular myocardium in young children whose coronary circulation is normal. Since the scar is weakest during the second week after the puncture, restriction of activity may be desirable.

Note: While the experiments were in progress, Seldinger[7] described a remarkably similar stylet catheter for splenic puncture in lienoportal venography and percutaneous transhepatic cholangiography. Earlier he had applied a similar instrument to the puncture of arteries and veins.[8, 9]

SUMMARY

A new method for percutaneous left ventricular puncture is described. A 19 gauge stylet threaded through a polyethylene or Teflon tubing was directed into the left ventricle through the left paraxiphoid angle in rabbits, and through the fourth left intercostal space anteriorly in dogs. The stylet or needle was then withdrawn, leaving the tubing in place in the ventricular cavity. The tubing was passed into the aorta and left atrium, and pressure tracings were taken. In rabbits, bleeding was negligible when the first attempt was successful; but its incidence and amount were increased greatly when more than one attempt was made. Microscopically, there was early scar formation and intact visceral pericardium and endocardium. In dogs the left ventricle was easier to puncture and no bleeding occurred. Direct visualization of multiple punctures of a dog's heart and pericardium showed that ventricular and small branch coronary artery bleeding were insignificant, while the pericardial vessels and atria might bleed enough to require pericardial tapping. As the procedure seemed reasonably safe to apply in infants and small children, it was performed in a 3-month-old infant severely ill with an incompletely diagnosed congenital anomaly of the heart. The solid stylet was replaced by a hollow-needle stylet. The stylet tubing was introduced at the apex. The left ventricle was entered, the left atrium and aorta were catheterized, and satisfactory cardioangiography was performed, without ill effects.

We wish to acknowledge the assistance with the histology provided by Dr. Serafin Juliano, ICA Fellow in Pathology, Department of Pathology, Indiana University School of Medicine. Mr. Clyde Foster gave valuable technical assistance. Dr. Harold King of the Department of Surgery originally suggested the idea of a stylet catheter to us.

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LABORATORY METHODS

QUANTITATIVE DETERMINATION OF FREE LYSINE IN URINE BY TWO-DIMENSIONAL PAPER CHROMATOGRAPHY

LUCY F. KERLEY, B.Sc., D.A.C., A.R.A.C.I.
MELBOURNE, AUSTRALIA

IN THEIR earlier surveys of amino acid excretion in various disorders Dent and associates[1, 2] failed to resolve lysine and ornithine and lysine and arginine. Since preliminary observations of amino acid excretion during pregnancy indicated that fluctuations of lysine concentration appeared greater than that of any of the other amino acids, conditions were sought under which this amino acid could be unequivocally determined.

The present modification was designed to give a quantitative determination of lysine by the optical scanning method using an "Eel" scanner.* This was achieved by (1) keeping the specimen at pH 2, enabling a standard to be run and measured on the same paper side by side with the unknown under, as nearly as possible, the same conditions (at this pH a compact spot of lysine dihydrochloride was obtained), and (2) enlarging the area of the applied sample, contrary to the generally accepted practice, to give a lysine spot large enough to be measured in the Eel scanner.

The use of the Dent apparatus allowed as many as 10 determinations to be carried out together.

MATERIALS AND METHODS

Apparatus.—The two-dimensional capillary ascent technique was used with stainless steel apparatus, as described by Datta and associates,[3] which allows minimum handling of the sheets.

Paper.—The paper used was Whatman No. 1.[4] Each sheet measured 12 by 12 inches.

Solvents.—Eighty per cent phenol in water was used as the first solvent. Since the method depends on a particular form of pH control, ammonia was not necessary. This also minimizes background color which has been shown to be responsible for high and variable blanks.[6, 7, 14] A.R. grade B.D.H.† phenol was found to be satisfactory without further purification, and the solution could be reused[7] as many as 4 times until a pink color developed.

The second solvent was a 65 per cent solution of a mixture of 2:4 and 2:5 lutidine (A.R. grade B.D.H.). A number of water-miscible solvents,[8] 60 per cent acetone, 75 per cent ethanol,

From the Department of Obstetrics and Gynaecology, University of Melbourne, and the Royal Women's Hospital, Melbourne, Australia.
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*Manufactured by Evans Electroselenium, Ltd., Harlow, Essex, England.
†Analytical reagent grade, British Drug Houses, Ltd., England.

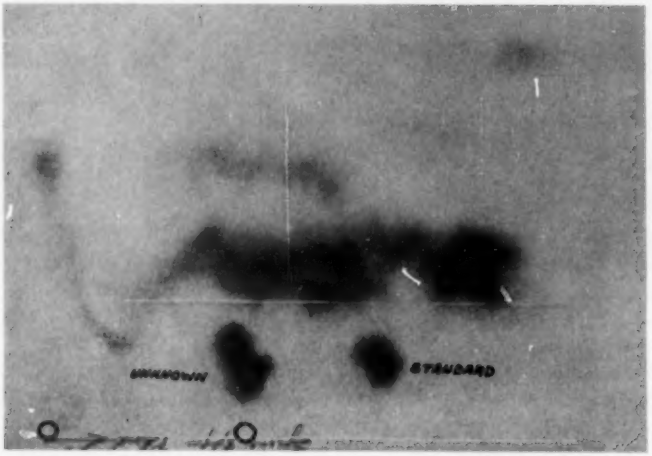

Fig. 1.—Illustrated are the final positions of lysine in the urine specimen (pH 2.2) and the standard lysine (pH 1.4) after completion of the two dimensional run. The strip cut to fit the scanner can be seen.

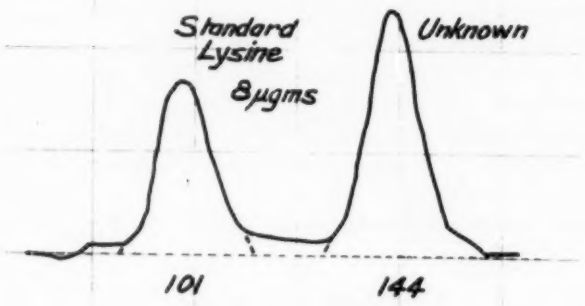

Fig. 2.—The optical density curves of the lysine spots shown in Fig. 1.

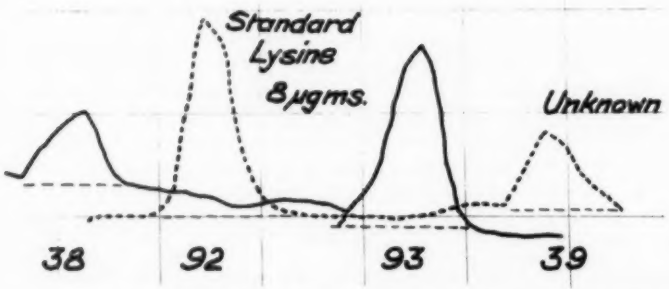

Fig. 3.—Scanner curves show result of reversing strip in holder to obtain base line.

n-butanol-acetic acid-water mixture (40:10:50), was tested, but they were found to be unsuitable.

Development of Spots.—One-tenth per cent ninhydrin in 95 per cent ethanol was sprayed on the sheets, which were allowed to dry overnight at room temperature to obtain maximum development of the colors.[9]

Specimens.—Twenty-four-hour urine specimens containing 50 ml. 1.5N HCl as preservative were each made up to 2 liters. To ensure that collection of the specimens was complete, creatinine was determined.

Desalting.—This[10, 11] was considered unnecessary as in the few cases where a "salt effect" was noticed the lysine spot was not affected.

Technique.—In order to obtain spots of a size suitable for measurement in the Eel scanner, volumes of urine somewhat greater than generally recommended were used.

One hundred microliters of the diluted specimen (pH 2) were placed at the lower left-hand corner of the paper, 1½ inches from the lower edge and 1 inch from the left-hand side. An 0.1 ml. micropipette was used to add the specimen a drop at a time. Each drop was dried immediately by means of an electric hair dryer to ensure the diameter of the applied spot to be about 1.5 cm.

It was found that this volume of urine gave approximately 2 to 10 μg of lysine, a conveniently measureable quantity. For the minimum quantity of lysine which gives visible colors with ninhydrin, varying figures are given dependent on the conditions.[12-14]

Eight microliters (1 μg per microliter) of standard lysine solution of pH 2 was delivered by means of a calibrated red blood cell micropipette at a point 3 inches to the right of the origin and allowed to spread to the same area as the unknown. Duplicate samples were treated with H_2O_2[12, 15] and lysine appeared to be unaffected.

The temperature of the chromatography room varied only from 20 to 22° C. The sheets were allowed to equilibrate overnight for at least 18 hours.[16] After the phenol run the sheets were dried at room temperature for about 3 hours and finally were transferred to a thermostatically controlled cupboard equipped with a fan, and dried at 35 to 40° C. for an additional 6 hours. By this time no smell of phenol remained.

The phenol is not easily removed at room temperature. On the other hand, if dried at high temperature losses of amino acids occur.[5, 14, 17, 18] Under our conditions the phenol is completely removed at a temperature which should result in a minimum loss of amino acids.

Since the standard and unknown lysine were run on the same sheet and their concentrations were of the same order of magnitude, any slight loss should not affect the final result which is a relative figure only.

After equilibration for 2 to 3 hours the lutidine solution was run for 24 hours.

The use of the basic solvent first[10] would reduce considerably the total running time of the chromatogram, since drying of the sheets after each run would be quicker. However, Block[19] found that under these conditions the color intensities were less than when phenol is used first.

After development with ninhydrin the piece of sheet containing the unknown and standard lysine spots was cut out to fit an Eel scanner (Fig. 1). The optical density was read on a calibrated microammeter, and the reading was plotted directly to give a graph of the concentrations. The areas under the two peaks were measured by counting the squares on the graph paper and the unknown calculated as milligrams per 24 hours (Fig. 2).

The Eel scanner is normally used to measure proteins after separation by electrophoresis. It is a simplification of an instrument such as that described by Grassmann and Hannig.[24]

The other constituents of the urine affect the standard less than they do the unknown. Other amino acids have been in contact with the paper between the unknown and the standard during the phenol run, and may have left a slight residue detectable by the scanner. In some cases the graph did not return to the original zero position. In an attempt to cancel out this effect, after scanning in the direction standard to unknown, the strip was reversed in the holder to measure in the opposite direction (Fig. 3).

The length of the slit of the scanner is 3½ cm. The originally applied spots of urine and of standard of diameter 1½ cm. gave lysine spots each of diameter 2½ cm. This left a blank margin of ½ cm. on the paper both above and below the spot. The length of the slit is fixed; therefore, we attempted to remove the effect of the blank margins by placing strips of black paper on the glass plates but the curves ran off the graph paper. It was found more satisfactory to leave the blank margins provided care was taken to keep them the same width for both unknown and standard. The results for two pairs of lysine spots of different concentrations, each pair run at different times, show this effect (Table I).

TABLE I

	(A) LARGER BLANK MARGINS		(B) SMALLER BLANK MARGINS	
Lysine in μg	5.6	11.2	1.6	4
Area in 1/10 inch squares	48	90	42	94

After replacing the cut-out piece of paper with transparent tape the whole sheet was then photographed in transmitted light both in color and in black and white.

Finally, in anticipation of fading, the individual spots on the sheets were outlined in pencil and a description of the color and relative strengths was written beside each spot.

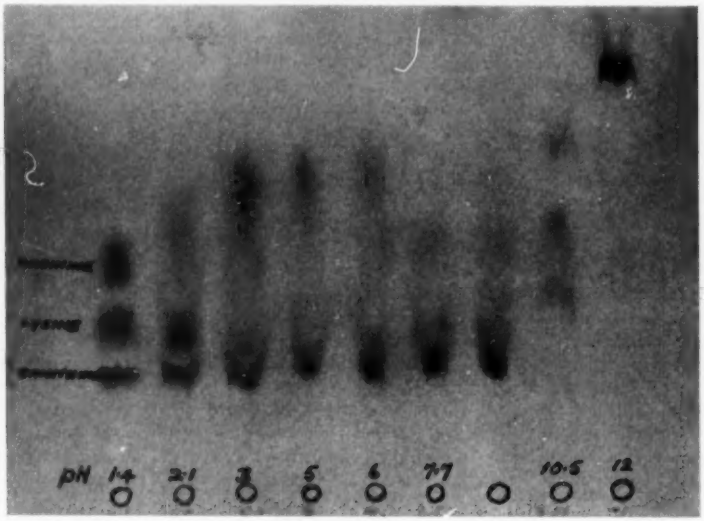

Fig. 1.—Four microgram mixtures of ornithine, lysine, and arginine at different pH's after phenol run.

RESULTS AND DISCUSSION

Arginine can occur in urine, and Stein[20] found large excretions of ornithine as well as lysine and arginine in the urine of 5 patients with cystinuria.

At pH 1 to 2 in a phenol-water system lysine gives a single compact spot of the dihydrochloride, while between pH 2 and 10 it gives long streaks due to dissociation of the carboxyl group.[21, 22] At pH 11 to 12 a single spot again occurs; since the two amino groups have lost their protons, this represents the anion. Arginine and ornithine behave in a similar fashion.

With a mixture of arginine, lysine, and ornithine, discrete spots appear at and below pH 2, but at pH 11 to 12 the 3 coincide at Rf 0.75. This behavior is shown in Fig. 4.

To ensure that the unknown spot being studied is, in fact, lysine, identification was established by adding known amounts of lysine, arginine, and ornithine to the urine sample for the two-dimensional run. Only the added lysine coincided with the unknown spot (Figs. 5 and 6).

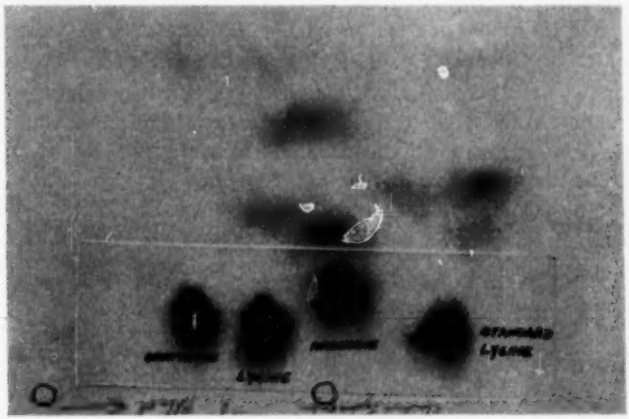

Fig. 5.—Chromatogram of urine specimen (pH 2) shows quantitative measurement of lysine in presence of ornithine and arginine. Eight micrograms each of arginine, lysine, and ornithine has been added to the specimen which originally contcontained no detectable lysine.

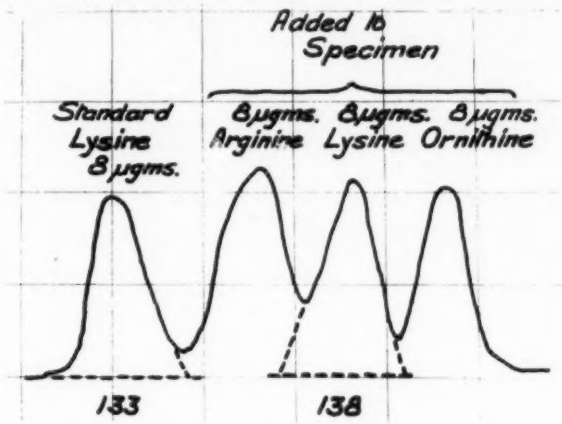

Fig. 6.—Scanner curves of spots shown in Fig. 5.

Landua and associates[22] suggest pH 10 as the most suitable condition for the separation of lysine. Our results show that this is not desirable when arginine or ornithine is present.

A pH of 1 to 2 is not necessarily the most suitable pH for other amino acids which occur in urine. At this pH some give rather large and, in some cases, diffuse spots, although preliminary experiments have shown that taurine, cystine, alanine, and glutamine may also be determined on the same paper as the other 3. Therefore, if amino acids other than these are to be determined, some other pH will be necessary or, alternatively, other solvents must be used.

Stein and Carey[23] have shown that the concentration of lysine in urine increases after acid hydrolysis. Our conditions are very mild compared with those necessary for hydrolysis, and no change has been observed after 8 months' storage in a refrigerator.

It cannot be stressed too much that in all chromatographic work it is imperative to know exactly the conditions under which the experiments have been carried out before any interpretation of a chromatogram is attempted. For example under the conditions of our experiments (pH 1 to 2) glutamic acid appears as a horizontally streaked spot above lysine (Fig. 5). Under alkaline conditions Dent and Walshe[2] have shown glutamic acid as a compact spot appearing in approximately the same position as lysine does under our conditions.

SUMMARY

A two-dimensional chromatographic method for the determination of lysine in urine has been described. A standard is run on the same paper. By adjustment of the pH of the samples and of the area over which they are allowed to spread when applied, spots of one lysine species are obtained which can be compared by means of an Eel scanner.

I wish to thank Miss M. Johnson (Pathology Department, Royal Women's Hospital) for the photographic work, and Mr. J. W. Legge (Biochemistry Department, University of Melbourne) for his helpful criticism during the writing of this paper.

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DIFFERENTIAL ULTRAVIOLET SPECTROPHOTOMETRIC DETERMINATION OF SERUM SALICYLATES

L. A. Williams, B.S.,* R. A. Linn, M.S.,* and B. Zak, Ph.D.**
Detroit, Mich.

THE quantitative determination of salicylates in biologic media has seen useful employment for clinical,[1] forensic,[4] and pharmacologic applications.[3] The determinative techniques have been mainly concerned with the phenolic nature of salicylate either through iron or metal reactions[6, 7, 9] or by coupling with diazotized *p*-nitroaniline.[5] Some investigation has been carried out on the reducing action of salicylates[8] or on the determination of salicylic acid in the ultraviolet region of the spectrum.[10] The problem of the isolation of salicylic acid for quantitative and qualitative ultraviolet spectrophotometry free from the interferences encountered in biologic material can be simplified by the use of the differential characteristics of the ultraviolet spectra of salicylate at two different pH levels. Previously, it has been shown that the spectrophotometric determination of barbiturates can be carried out by the employment of automatic recording differential spectrophotometry.[12] This has provided a simple, rapid, and accurate ultraviolet spectrophotometric quantitation procedure in which a composite spectrum could be obtained. This spectrum represented the automatic subtraction of two spectra, both containing the same substance in the same concentration but at two different pH levels. The conversion of the solution from pH 13 to pH 10.5 resulted in a hypsochromic shift of the spectrum. The subsequent subtraction of one spectrum from the other spectrum showed characteristic differences peculiar to the barbiturate being investigated.[11]

The present investigation describes the use of an automatic ratio recording spectrophotometer for the quantitative determination of the differential spectral characteristics of salicylate at two pH levels. As previously described for barbiturates,[12] it obviates the time and error involved in manually determining spectra which are crude and limited in nature[2]; it does not use blanks since the two aliquots of salicylate blank each other just as if the spectra had been individually recorded against their own blanks; it eliminates subtraction of one spectrum from its mate since the instrumentation technique automatically substracts the two spectra, and it enables one to analyze for salicylate or barbiturate or a mixture of the two as they may be encountered in biologic media. The extension of the procedure to 100 μl samples for salicylate determination

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*Office of the Wayne County Medical Examiner, Detroit, Mich.
**Department of Pathology, Wayne State University College of Medicine, and Detroit Receiving Hospital.

is also described. The automatic ratio recording spectrophotometer is not necessary to the procedure since a manual spectrophotometer can also be used. However, automatic instrumentation effects a great saving in the time and effort involved in the technique to be described.

MATERIALS AND METHODS

Reagents.—

1. Chloroform, spectranalyzed or redistilled.
2. Sodium hydroxide, 0.45 N.
3. Ammonium chloride, 16 and 20 per cent.
4. Hydrochloric acid, 6 N.

Equipment.—

1. Beckman DK-2 automatic ratio recording spectrophotometer.
2. Silica cuvettes, macro- and micro-, 1 cm. light paths.

Instrumentation.—Zero the instrument, utilizing the hydrogen lamp source and the photomultiplier tube at position IX, using the following specifications:

Wave length	400 mμ
Approximate slit width	0.04 mm.
Sensitivity	100
Time	2 minutes
Time constant	0.2
Initial range	0 to 100
Scale, initial	per cent T
Final range	−0.3 to +0.7
Scale, final	absorbance

After zeroing the instrument, change the range to −0.3 to +0.7 and the scale to absorbance with the cuvettes in the cuvette holder. The pen will now automatically move to 0.3 absorbance on the chart paper. If the pen is not exactly at 0.3 absorbance, adjust it with the 100 per cent control knob. Record the spectra from 400 mμ to 230 mμ using the previously specified conditions.

Analysis of Unknown Solutions (Macromethod).—Pipette 1 ml. of serum (urine, or use a weighed portion of tissue homogenate) into a separatory funnel. Add 1 ml. of 6 N hydrochloric acid and 50 ml. of chloroform. Extract the acidified material for 5 minutes. Filter the chloroform layer into a clean dry separatory funnel, discarding the aqueous phase. Add 7 ml. of 0.45 N sodium hydroxide to 45 ml. or more of the chloroform solution of salicylate and back extract the chloroform phase for 5 minutes. Discard the chloroform layer and centrifuge the aqueous phase. Place two 3 ml. portions of the aqueous phase into two silica cells. To one cell add 0.5 ml. of 0.45 N sodium hydroxide and to the other add 0.5 ml. of 16 per cent ammonium chloride. Mix well, cover, and read differentially using the ammonium chloride treated sample in the reference side.

Analysis of Unknown Solutions (Micromethod).—Pipette 0.1 ml. of serum (urine or a weighed portion of tissue homogenate) into 10 ml. of chloroform. Add 0.1 ml. of 6 N hydrochloric acid and extract for 5 minutes. Centrifuge, carefully aspirate off the upper layer, and discard the aspirated material. Pipette 1.0 ml. of 0.45 N sodium hydroxide into a 9 ml. aliquot of the chloroform solution and back extract for 5 minutes. Centrifuge and carefully remove the lower layer using a long needle on a syringe. Pipette 0.4 ml. aliquots of the alkaline extract into two microcuvettes and then add 0.05 ml. of 20 per cent ammonium chloride to one cuvette and 0.05 ml. of 0.45 N sodium hydroxide to the other cuvette. Mix and read differentially using the conditions previously described, and with the smallest masking slit of the DK-2 filter holder turned parallel to the instrument slit.

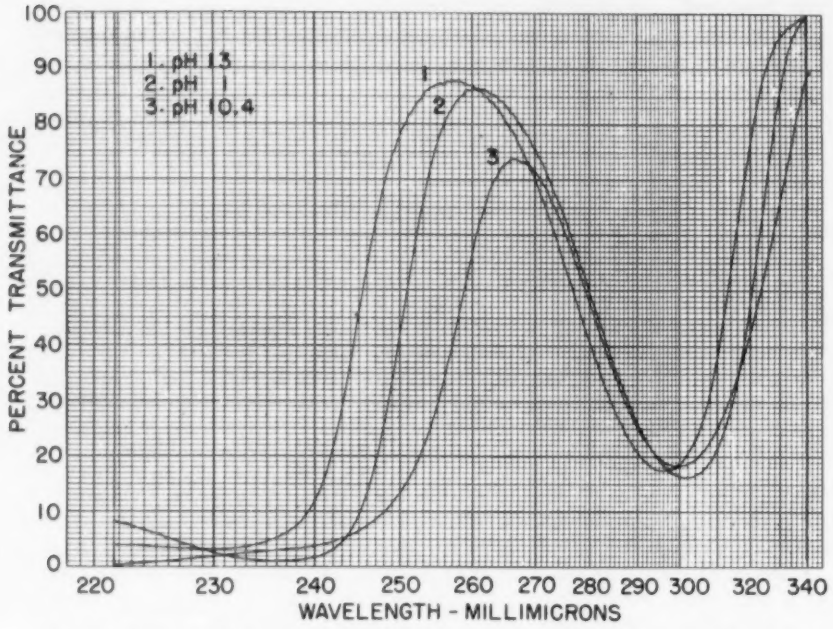

Fig. 1.—Wave length per cent transmittance spectra for salicylate at various pH levels.

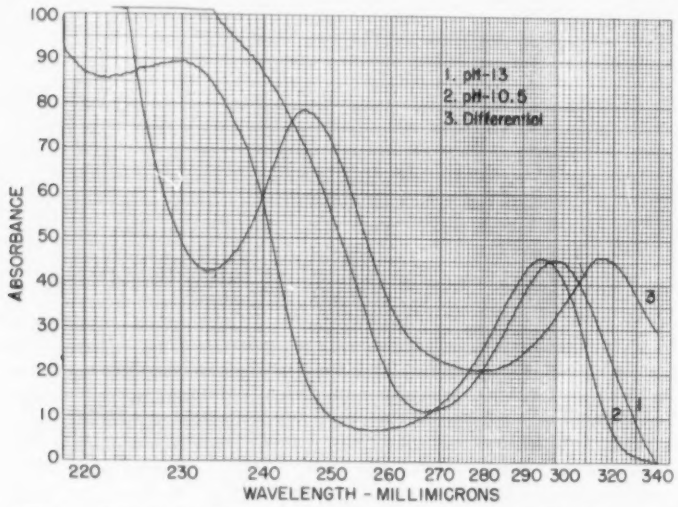

Fig. 2.—Normal and differential spectra of salicylate at pH 10.5 and pH 13.

RESULTS AND DISCUSSION

In order to determine the two pH levels which would give adequate analytic sensitivity as well as distinguishing qualitative characteristics, a number of spectra were graphed at various pH levels. The extremes are shown in Fig. 1. These transmittance wave length curves were made with 4.3 μg of salicylate per millimeter and recorded against their appropriate blank. It is quite evident that the best differential curve could be obtained by the subtraction of the readings of Curve 3 from Curve 1. Curve 1 was carried out in 0.45 N NaOH, Curve 2 in 0.1 N H_2SO_4, and Curve 3 in NaOH and NH_4Cl buffer of pH 10.4. These happen to be ideally suited since they are also the best pH's for the similar determination of barbiturates.

Fig. 2 shows the spectra obtained with salicylates using the two pH levels decided upon. Curve 1 shows the pH 13 graph, Curve 2 shows the pH 10.5 graph, and Curve 3 shows the composite spectrum obtained by using the differential characteristics of the instrument to subtract one curve from the other as described in materials and methods. The two maxima occur at 318 and 246 mμ while the two minima occur at 283 and 233 mμ, respectively. Since both cuvettes contain equal aliquots of the same extract material and are distinguished from each other only by pH difference, interference from extraneous ultraviolet absorbing material can only occur if the interfering compound also shows a similar hypsochromic spectrum shift at the two pH values employed. This represents a unique advantage over normal ultraviolet analysis of biologic material since there are many compounds capable of exhibiting ultraviolet spectral characteristics which could show an additive error on the desired constituent and which could not show the characteristic shift with pH. Thus, graphing one against the other would yield a straight line for this material with no interference on the differential spectrum.

Fig. 3 shows a series of differential absorbance wave length spectra for various concentrations of salicylate. The range extends from 4 mg. per 100 ml. for Curve A to 55 mg. per 100 ml. for Curve F. It can be seen that the 318 mμ peak could be usefully employed for high concentrations of salicylate whereas the peak at 246 mμ would be suitable for lower concentrations of salicylate. The latter peak has a sensitivity of 3 times that of the peak at 318 mμ. The minimum at 283 mμ is of a lower order of sensitivity than is the maximum at 318 mμ and could be used for salicylates above the 70 mg. per 100 ml. level.

Fig. 4 shows the calibration curves obtained using the absorbance differences at the 3 wave lengths. The 246 mμ calibration plot is sensitive and useful from 0 to 20 mg. per 100 ml. while the 318 mμ plot covers the range of 0 to 70 mg. per 100 ml. Above 70 mg. per 100 ml., one could use the 283 mμ curve although the order of sensitivity is lower.

Table I shows a series of representative recoveries may by extracting absolute amounts of salicylate from pure solution as well as from additions to sera which had been shown to be salicylate free. In comparing the differential

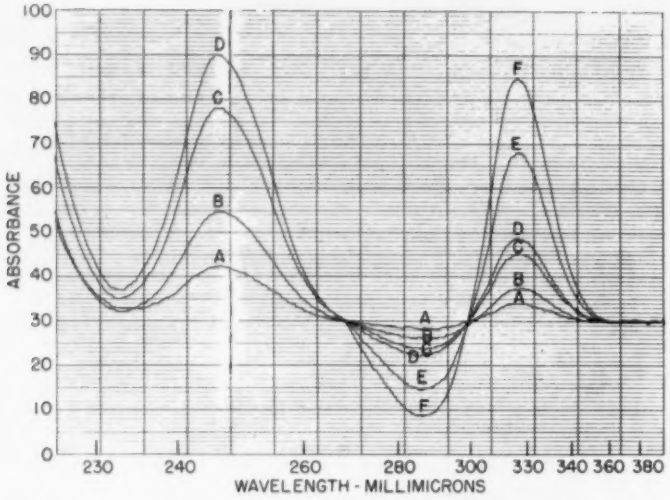

Fig. 3.—Differential absorbance wave length graphs of several concentrations of salicylate.

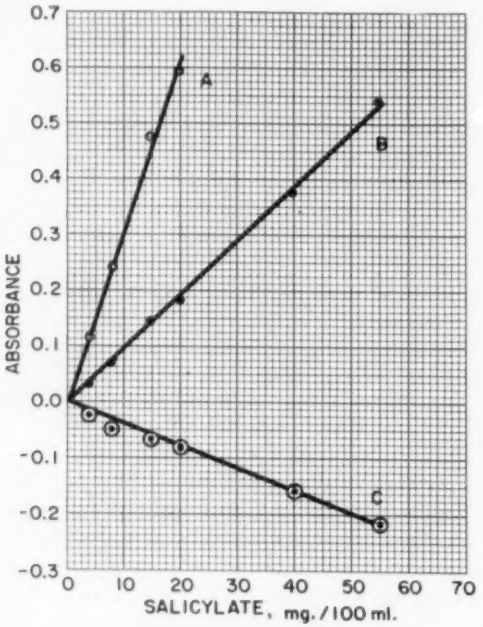

Fig. 4.—Calibration curves for differential spectra of salicylate at its minimum and maximum absorbance peaks.

spectra of salicylate extracted from sera and water, a reasonable similarity was obtained between the pure solution and the matrix investigated. The accuracy of the recovery of these comparisons and those in Table I were excellent.

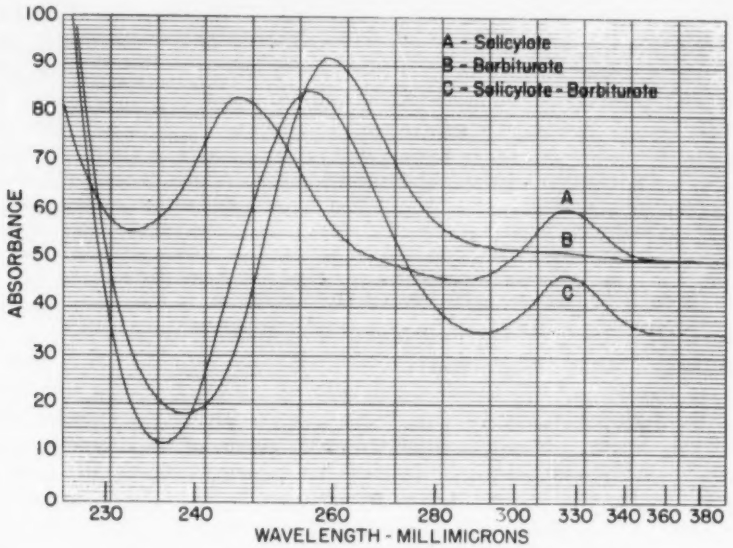

Fig. 5.—Curve A, salicylate differential spectrum, Curve B, seconal differential spectrum, Curve C, differential spectrum of a mixture of barbiturate and salicylate.

TABLE I. RECOVERY OF ABSOLUTE ADDED AMOUNTS OF SALICYLATE FROM WATER AND SERUM

SAMPLE	SALICYLATE PRESENT (MG./100 ML.)	SALICYLATE FOUND (MG./100 ML.)
Serum A	10.0	10.0
Serum B	20.0	21.0
Serum C	30.0	30.2
Serum D	40.0	40.6
Serum E	15.0	14.0
Serum F	10.0	10.5
Serum G	14.0	14.5
Serum H	20.0	20.8
Serum I	30.0	28.0
Serum J	40.0	44.6
Standard 1	10.0	10.0
Standard 2	10.0	10.2
Standard 3	20.0	19.7
Standard 4	30.0	29.4
Standard 5	30.0	29.2
Standard 6	40.0	43.2

Curve B of Fig. 5 shows the characteristic nature of the composite spectrum one obtains in this case by differential spectrophotometric means with a common barbiturate derivative. Curve A represents a similar consideration for salicylic acid, i.e., a composite differential spectrum with a pH 10.5 solution of salicylate in one cuvette and a very alkaline solution of salicylate in the other (0.45 N NaOH). It would not be difficult to recognize whether one or the other were

present if they were present as a single contaminant of serum or tissue. If one were to be confronted with the prospect of both being present at the same time, Curve C would be the resultant composite spectrum obtained using the described differential technique. The height of the first differential salicylate peak appears unimpaired by the contaminant whereas the next two absorbance peaks, both negative and positive, are so affected as to be inaccurate. Re-extraction of a second portion of the investigated material at pH 7.4 leaves the salicylic acid unextracted while the barbiturate is completely removed to the chloroform phase. Thus, one is able to analyze a mixture of both salicylate and barbiturate if they are present in the same physiologic matrix. More commonly they occur as individual entities, and the procedure is simplified. However, salicylate can be analyzed in the presence of barbiturate with good accuracy, and this would be more difficult to accomplish by conventional ultraviolet spectrophotometry.

SUMMARY

A procedure has been described for the determination of salicylate which involves the use of the differential characteristics of an automatic ratio recording spectrophotometer. The technique obviates normal blanking and eliminates interference from ultraviolet absorbing material. The instrument automatically subtracts two spectra of salicylate, one from the other, after a hypsochromic shift with a pH change and draws a composite spectrum. The analysis range is wide, the procedure is applicable to 100 μl samples, and barbiturate, when present, can be determined regardless of whether salicylate is also present or entirely absent.

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NATURALLY ACQUIRED TOXOPLASMOSIS IN THE GRAY SQUIRREL, SCIURUS GRISEUS, AND ITS BEARING ON THE LABORATORY DIAGNOSIS OF RABIES

ORLAND A. SOAVE, D.V.M., AND EDWIN H. LENNETTE, M.D.
BERKELEY, CALIF.

IN NOVEMBER, 1957, the carcass of a gray squirrel (*Sciurus griseus*) was sent to this laboratory with a request that the brain be examined for evidence of infection with rabies virus. The animal had been noted to behave in an abnormal manner and had bitten a child. Because of this episode, the animal was impounded; shortly after its capture, it became comatose and died on the following day.

Laboratory examination revealed no evidence that the squirrel had succumbed to rabies but, rather, that death might possibly have been due to toxoplasmosis. Findings bearing upon this latter point form the basis of this report, which is presented because of the almost complete absence of information on naturally occurring Toxoplasma infection in squirrel species, and because toxoplasmosis is seldom considered in the examination of tissue of wild animals showing overt evidence of C.N.S. disease.

MATERIAL AND METHODS

Preparation of Inoculum.—A small portion of the squirrel brain was weighed and ground manually in a mortar until a fine paste was obtained; sufficient 0.85 per cent saline solution was added incrementally to give a 10 per cent suspension. This suspension was centrifuged horizontally at 2,500 r.p.m. for 10 minutes and the supernatant fluid carefully removed. To each 0.9 ml. of fluid was added 0.1 ml. of an antibiotic solution containing 10,000 units per milliliter of penicillin G and 50,000 units per milliliter of streptomycin sulfate. The mixture was incubated at 4° C. for approximately one hour and then inoculated intracerebrally in 0.03 ml. amounts into mice 3 to 4 weeks old.

Microscopic Examination.—Smear and imprint preparations were made from the squirrel brain, utilizing tissue from the region of Ammon's horn. The preparations were stained by Seller's method, which is routinely employed in this laboratory for the staining of Negri bodies. Subsequently, when the presence of toxoplasms was suspected, additional smear and imprint preparations were made and stained by the Giemsa method.

RESULTS

Comprehensive examination of numerous microscopic fields of the slides stained by Seller's method failed to reveal the presence of any structures identifiable as Negri bodies. Examination of these slides with an oil immersion objective revealed the presence of a number of large round structures about 30 to 50 μ in diameter and containing numerous small bodies of 2 to

From the Viral and Rickettsial Disease Laboratory, California State Department of Public Health, Berkeley 4, Calif.
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3 μ in size. These small bodies had a pale-blue color and presented little or no nuclear detail; the characteristic appearance of these structures, and their collection into an aggregate surrounded by a barely discernible membrane, led to their morphologic identification as *Toxoplasma gondii* in the pseudocyst form. Occasional free forms of this organism were encountered throughout the brain smears. These forms had a crescent-shaped or irregular appearance and occurred singly or in groups of two or three.

The microorganisms were more clearly visible and readily distinguishable when stained by the Giemsa technique (see Fig. 1). In such preparations, the toxoplasms had a pale magenta color and appeared as rounded bodies with a slightly darker staining nucleus. As in the case of material stained by Seller's method, the limiting membrane of the pseudocyst was not clearly visible.

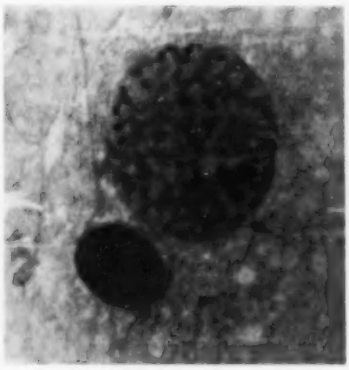

Fig. 1.

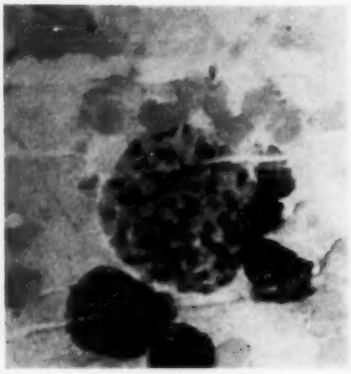

Fig. 2.

Fig. 1.—Toxoplasmal pseudocysts in brain of naturally infected squirrel, *Sciurus griseus*. Touch impression, Giemsa stain. (Magnification ×1125.)

Fig. 2.—Toxoplasmal pseudocysts in brain of albino Swiss mouse inoculated with suspension of squirrel brain. Smear preparation, Giemsa stain. (Magnification ×1125.)

Isolation of *T. gondii* is usually done by inoculation of heavy suspensions of fresh tissue into mice (or other laboratory animals) by the intraperitoneal or intracerebral routes. Since, in the present work, diagnostic attention was focused upon isolation of rabies virus, the suspension of squirrel brain for mouse inoculation purposes was prepared according to basic virologic procedures, i.e., it was centrifuged at a sufficient number of g to throw down the large tissue particulates and much of the gross cellular debris. Such treatment serves, in effect, to sediment protozoan parasites such as *T. gondii* and since the supernatant fluid is used for animal inoculation, recovery of the toxoplasm is precluded or made highly difficult. Of the 8 mice inoculated intracerebrally with the supernatant fluid obtained by centrifugation of squirrel brain suspension, one died on the eleventh postinoculation day and another appeared ill and was killed on the fifty-second postinoculation day. Imprint

preparations from the brain of both animals showed the presence of toxoplasmal pseudocysts; Fig. 2 shows the structures seen in the brain of the first mouse.

DISCUSSION

Since the original reports of Nicolle and Manceaux[1] from North Africa and of Splendore[2] in Brazil describing Toxoplasma as a new protozoon from the gondi (*Ctenodactylus gondi*, a rodent) and from the domestic rabbit, respectively, the parasite has been described from a wide variety of mammalian hosts, from some marsupials, and from certain avian species. Many of these descriptions of natural infection are based on morphologic observations and hence may be open to question; although the appearance of *T. gondii* is rather distinctive, an unknown organism cannot be identified definitively as *T. gondii* on the basis of morphology alone.[3] In addition to possessing the morphologic and tinctorial characteristics of *T. gondii*, the unknown microorganism should be shown to be an obligate intracellular parasite, to possess pathogenicity for mice and other laboratory species, and to be immunologically related to known strains of *T. gondii*. Unfortunately, because of the pressure of other and more urgent work, no attempt was made to fulfill these criteria. One important consideration, based on the results of inoculation in the first passage mice, and perhaps unwarranted, was that *T. gondii* may acquire pathogenicity only after prolonged passage at frequent intervals, and that some strains, even after a year or more of continual passage at frequent intervals, still retain minimal levels of virulence.[4-6] Thus, Pope and associates[5] describe a strain which produced only inapparent infections in mice during the first 64 intraperitoneal passages; thereafter the virulence for mice gradually increased, and from the eighty-fifth passage onward the mortality was 100 per cent.

Although the diagnosis of toxoplasmosis in the tree squirrel, *Sciurus griseus*, reported upon here is based entirely upon the tinctorial and morphologic characteristics of the microorganism seen in tissue preparations, we considered it desirable to record the findings since the only other instance of natural toxoplasmosis in the squirrel (red squirrel) of which we are aware is that recorded by Coles[7] in 1914. In addition, the experience recorded here suggests that *T. gondii* should be considered as a possible etiologic agent in the production of those symptoms that lead to subsequent examination of the animal for rabies virus infection, and might especially be considered when the results of such examination prove negative. This possibility finds support in recent reports[8, 9] from North Africa that the squirrel *Citellus citellus* is highly susceptible to experimental toxoplasmosis.

SUMMARY

The brain of a California gray squirrel (*Sciurus griseus*), a tree-living species, was examined for rabies virus because the animal had been observed to behave in an abnormal manner and had bitten a child. Microscopic examination of smear and imprint preparations of the brain was negative for

Negri bodies but structures with the typical morphologic and staining characteristics of *T. gondii* were encountered. This is believed to afford at least presumptive evidence of natural infection with *T. gondii* in a squirrel.

The excellent technical assistance of Mr. Koichi Nakamura is gratefully acknowledged.

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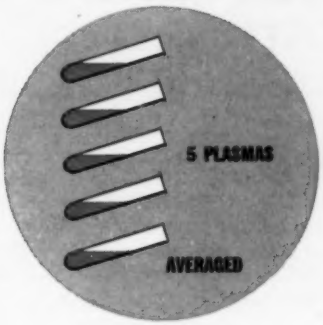

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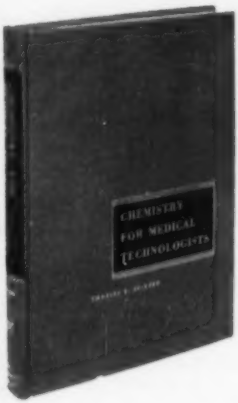

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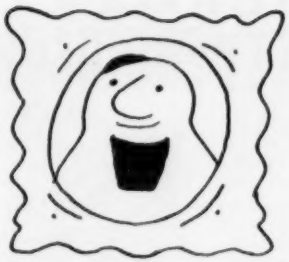

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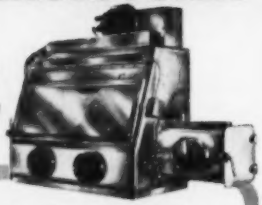

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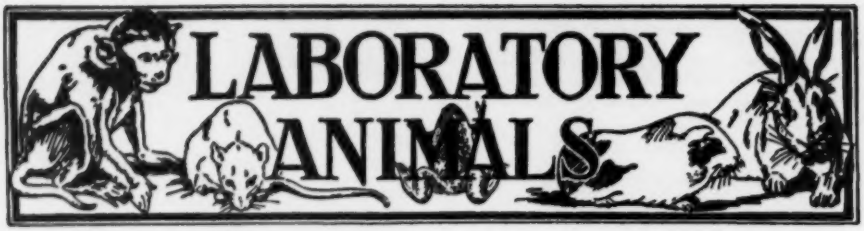

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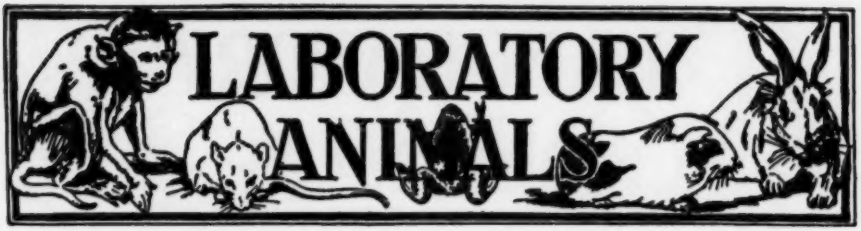

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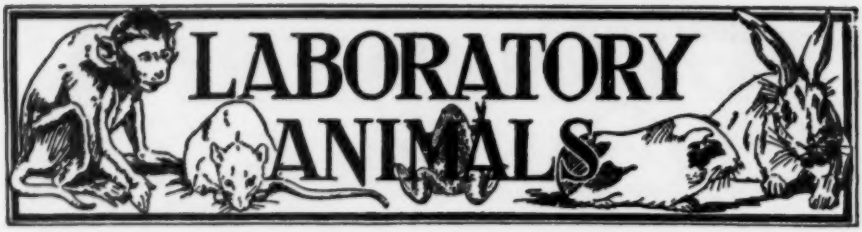

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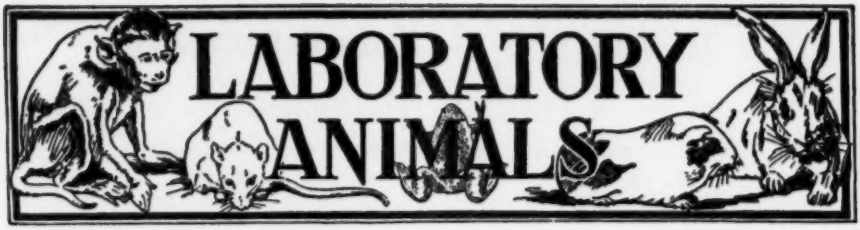

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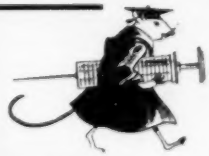

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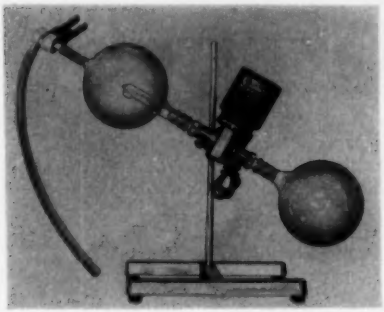

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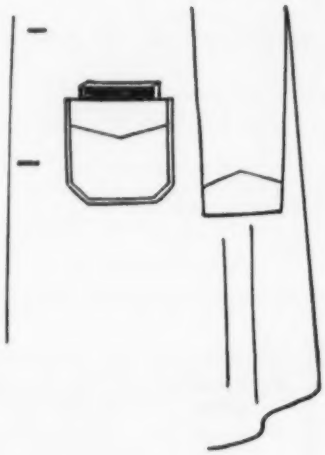

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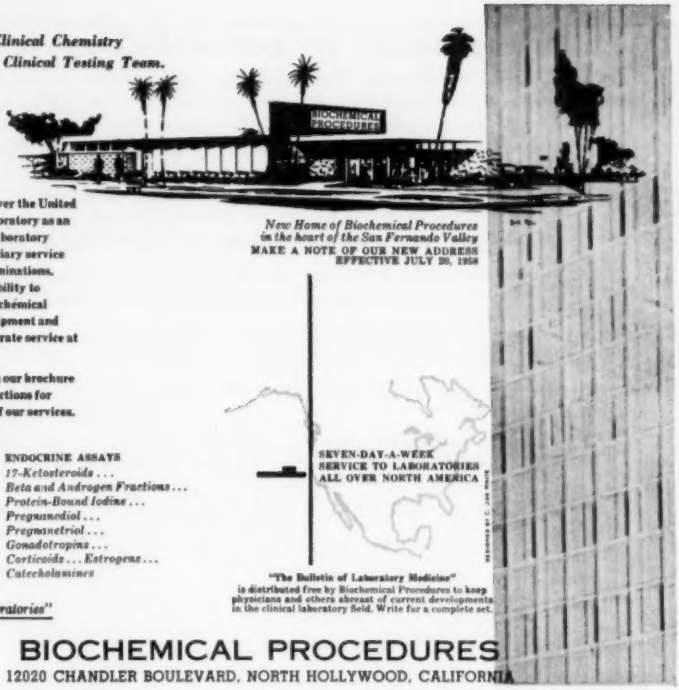

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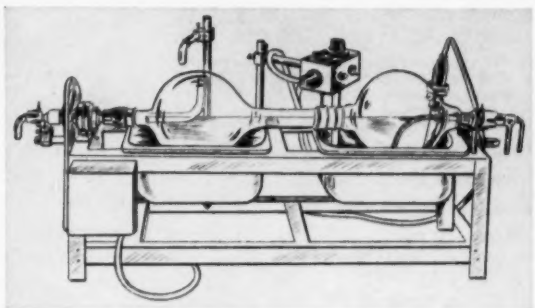

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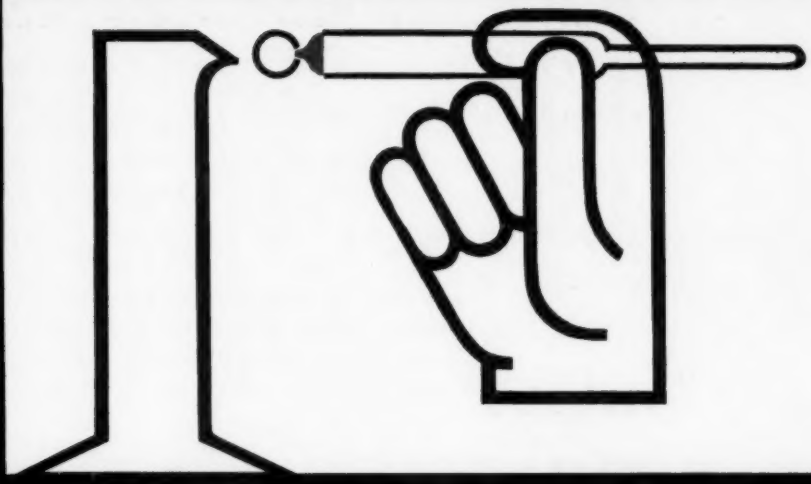

What is the most practical approach to determining the concentration of solids in urine?

Specific gravity. Increased output of urinary solids raises the specific gravity—each 1 per cent of glucose increases the reading by 0.004.

Source—Wolman, I. J.: Laboratory Applications in Clinical Pediatrics, New York, The Blakiston Division, McGraw-Hill Book Company, Inc., 1957, pp. 343, 344.

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*Giordano, A. S.; Pope, J. L., and Hagan, B.: Am. J. M. Technol. 22:29, 1956.

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